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(54) Title: CATIONIC DRUGS ENCAPSULATED IN ANIONIC LIPOSOMES

(57) Abstract

Some cationic drugs are effectively impermeable to cells, a factor that, along with toxicity, precludes their use against many important intracellular pathologies. Encapsulation of the drugs in non–cationic liposomes increased permeability of the drugs to cells and therefore widened the drugs' therapeutic spectrum. The present invention provides for cationic drugs encapsulated in non–cationic fusogenic liposomes, methods of treating pathological conditions with such liposomal–encapsulated drugs, and pharmaceutical compositions comprising such liposomal–encapsulated drugs.

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CATIONIC DRUGS ENCAPSULATED IN ANIONIC LIPOSOMES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Provisional Patent Application 60/069,359, filed December 12, 1997 which is incorporated by reference in its entirety.

FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Intracellular pathology constitutes a challenge for classical medicinal therapies because, to be effective against the pathological condition, the therapeutic agent must be internalized by infected cells. Many drugs active *in vitro* or extracellularly are often inactive inside a cell due to their poor penetration into cells or to their inactivation by lysosomal enzymes. Thus, the development of new therapeutic formulations or carriers capable of intracellular delivery of drugs will improve therapy for intracellular pathologies presently difficult to treat.

It is well recognized in the medical field that the most effective procedure for treating localized disease is to direct the pharmaceutical or drug agent (hereinafter "drugs") to the affected area, thereby avoiding undesirable toxic effects of systemic treatment. The same is true for drugs which affect intracellular processes. Typically, drugs enter cells by diffusion through the plasma membrane or are taken into the cell by pinocytosis, phagocytosis and endocytosis. Another, and perhaps more effective delivery system, uses liposomes containing the appropriate drug or chemical. The liposome fuses with the plasma membrane, thereby releasing the drug into the cytosol. Alternatively, the liposome is phagocytosed or taken up by the cell in a transport vesicle. Once in the endosome or phagosome, the liposome either degrades or fuses with the membrane of the transport vesicle and releases its drug. This latter step is the most problematic and, in fact, the greatest barrier

to the use of liposomes as drug carriers is formulating liposomes so that they release the drugs within a cell.

Current methods of drug delivery via liposomes require that the liposome ultimately becomes permeable and releases the encapsulated drug at the target tissue or cell. For systemic or tissue specific delivery, this can be accomplished, for example, in a passive manner wherein the liposome bilayer degrades over time through the action of various agents in the body. Every liposome composition will have a characteristic half-life in the circulation or at other sites in the body and, thus, by controlling the half-life of the liposome composition, the rate at which the bilayer degrades can be somewhat regulated.

In contrast to passive drug release, active drug release involves using an agent to induce a permeability change in the liposome vesicle. Liposome membranes can be constructed so that they become destabilized when the environment becomes acidic near the liposome membrane (*see, e.g., Proc. Natl. Acad. Sci. USA* **84**:7851 (1987); *Biochemistry* **28**:908 (1989)). When liposomes are endocytosed by a target cell, for example, they become destabilized and release their contents. This destabilization is termed fusogenesis.

Fusion of liposomes with cell and/or organelle membranes has been the subject of intense effort in the recent past. Dioleoylphosphatidylethanolamine (DOPE) is the basis of many "fusogenic" systems. This lipid does not form a bilayer when dispersed in aqueous media, preferring the hexagonal (H_{II}) phase. DOPE can be formulated into liposomes through addition of bilayer-stabilizing lipids. If these stabilizing lipids have negatively-charged head groups, upon acidification of the endosome the charge is neutralized by a proton, the stabilizing effect is lost and the DOPE begins to form hexagonal phase. The mechanism of fusion of liposomes with cell membranes is somewhat uncertain, but it appears that the H_{II} phase is an intermediate and hence this destabilization can theoretically promote fusion and release of liposomal contents into the cell cytosol. In spite of the intense efforts in this area, no one had reported the successful intracellular targeting of cationic drugs with fusogenic liposomes. Thus, there still exists a need in the art for a method for targeted drug delivery that overcomes the disadvantages of the currently available methods. Specifically, a parenteral delivery system is required that would allow retention of encapsulated or associated drug or therapeutic agent(s) within the liposome during circulation in the treated organism. This delivery system would be capable of delivering therapeutic agents or drugs within a target cell by active targeting (*e.g.*, by incorporating an antibody or cell-specific hormone on the surface of the liposomal vehicle)

or by passive targeting, as seen for long-circulating liposomes. Alternatively and preferably, the liposome could be taken up by the cell and the drug released intracellularly. Only specific cells (or bacteria, virions, enzymes, or other cellular mechanisms within the cell) would be susceptible to the drug. Following endocytosis of the liposome by the target cell, the liposomal carrier would become fusogenic and would release any encapsulated or associated drug or therapeutic agent in the target cell. Alternatively, the liposome would fuse with the target cell plasma membrane thus introducing the drug or therapeutic agent into the cell cytosol. In this way, cationic drugs, such as proteins, peptides, *etc.*, which are generally not permeable to the cell membrane, would be delivered to its required intracellular site of action. Quite surprisingly, the present invention provides such a targeting drug delivery system.

SUMMARY OF THE INVENTION

The present invention provides for an intracellular delivery system for cationic drugs. The drugs are encapsulated in non-cationic fusogenic liposomes. The fusogenic character of the liposomes is such that at physiological conditions, the liposomes maintain a bilayer structure. However, in an acidic environment, such as in an endosome, the liposome undergoes fusogenesis and releases its drugs.

In a preferred embodiment, the cationic drug is an anti-infective agent or an antineoplastic agent. In a more preferred embodiment, the cationic drug is an antibiotic. In a particularly preferred embodiment, the antibiotic is an aminoglycoside. In a most preferred embodiment, the aminoglycoside is gentamicin. It is contemplated that the drugs can either be on the exterior surface of the liposome or in the interior of the liposome. In a most preferred embodiment, the drug to lipid ratio is from about 0.05 to 0.25 by weight, more preferably from about 0.15 to 0.22.

In a preferred embodiment, the liposome comprises an anionic lipid. In a particularly preferred embodiment, the anionic lipid is R-dioleoylphosphatidylethanolamine, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl. In another preferred embodiment, the liposome further comprises a lipid selected from the group consisting of dioleoylphosphatidylethanolamine, diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, lysophosphatidylcholine,

diacylphosphatidylcholine, and dioleoylphosphatidylserine. In a particularly preferred embodiment, the liposome comprises dioleoylphosphatidylethanolamine (DOPE). In a more preferred embodiment, the percentage of DOPE is from about 20% to 90% and the percentage of anionic lipid is from about 10% to about 50%.

In a presently preferred embodiment, cholesterol is added to the liposome formulation for *in vivo* delivery of the cationic drug. The percentage of cholesterol added to the liposome is from 20% to about 50%.

To prevent aggregation during the formation of liposomes, a PEG-lipid derivative is added to the formulation. In a preferred embodiment, the anti-aggregant is a PEG-C_{20} ceramide or PEG-C_{14} ceramide, added at between about 0.25 to 5% (mol:mol).

In another aspect of the invention, methods for intracellular delivery of cationic drugs to ameliorate or treat a pathological condition are embodied. In a preferred embodiment, the cationic drug is an antibiotic. In a particularly preferred embodiment, the antibiotic is gentamicin. It is contemplated that the method encompasses drugs which are in the interior of the liposome. In a more preferred embodiment, the drug to lipid ratio is from about 0.05 to about 0.25 by weight, and more preferably from about 0.10 to 0.22.

In a preferred embodiment, the delivery method is by liposomes comprising an anionic lipid. In a particularly preferred embodiment, the anionic lipid is R-dioleoylphosphatidylethanolamine, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl. In another preferred embodiment, the liposome also comprises a lipid selected from the group consisting of dioleoylphosphatidylethanolamine, diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, lysophosphatidylcholine, diacylphosphatidylcholine, and dioleoylphosphatidylserine. In a particularly preferred embodiment, the liposome comprises dioleoylphosphatidylethanolamine (DOPE). In a more preferred embodiment, the percentage of DOPE is from about 20% to about 90% and the percentage of anionic lipid is from about 10% to about 50%.

In a presently preferred embodiment, cholesterol is added to the liposome formulation for *in vivo* delivery of the cationic drug. The percentage of cholesterol added to the liposome is from 20% to about 50%.

To prevent aggregation during the formation of the liposome, a PEG-lipid derivative is added. In a particularly preferred embodiment, the PEG-lipid derivative is a PEG-C_{20} ceramide.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. Removal of externally-bound gentamicin from anionic liposomes. DOPE:n-succinylDOPE:PEG (69.5:30:0.5 mol%) liposomes were formulated with gentamicin (filled bars) or formulated empty and exposed to gentamicin to allow binding to the exterior surface (open bars). These liposomes (100 μL at 40 mg/mL lipid) were passed through a SEPHADEX G50 column pre-equilibrated at either pH 7.4 or pH 10 and the post-column drug/lipid ratio subsequently determined by dual-label scintillation counting.

FIGURE 2. Retention of gentamicin in liposomes. Liposomes containing gentamicin were dialyzed against (A) HBS pH 7.4 and (B) CAB pH 4.5 for 22 h at 37°C. Aliquots were removed at various times, passed through SEPHADEX G50 columns and drug/lipid ratios were determined by dual-label scintillation counting. Squares: DPPC:Chol:DODAC (30:40:30 mol%); Circles: DOPE:n-succinyl-DOPE:PEG (69.5:30:0.5 mol%).

FIGURE 3. Uptake of liposomes to J774 cells. Liposomal formulations of gentamicin (1 μmol/mL; 200 μL) were incubated with $5x10^5$ J774 cells for 2 h at either 37°C or 4°C. Excess liposomes were washed off, the cells were collected and the amount of liposomes associated with the cells was determined by scintillation counting. Uptake for each formulation was quantitated by subtraction of the 4°C result (binding only) from the 37°C result (uptake plus binding). A:DPPC:Chol:DODAC (30:40:30); B: DPPC:Chol:DOPS (30:40:30); C:DOPE:n-succinylDOPE (70:30); D:DOPE:n-succinylDOPE:PEG (69.5-30:0.5); E:DOPE:n-succinylDOPE:PEG (69:30:1); F: DOPC:n-succinylDOPE (70:30).

FIGURE 4. Killing of intracellular wild-type (A) or hemolysin-expressing (B) *Salmonella typhimurium* by liposomal gentamicin. Results shown are representative assay results from a single experiment of multiple replicates. Each result represents a mean of triplicate assays performed together, with standard deviations. Antibacterial activity is expressed as percentage killing of intracellular bacteria compared to control cells that received identical treatment to test cells at the same time, with the

exception of the addition of liposomes. J774 cells infected with bacteria were incubated with liposomal gentamicin formulations at a gentamicin dose of 130.5 μg/mL for two hours. Cells were lysed and the lysate plated on growth medium overnight. Bacterial colonies were counted and the results were expressed as percentage killing compared to control cells that received no liposome treatment. A:DOPE:n-succinylDOPE (70:30); B:DOPE:n-succinylDOPE:PEG (69.5:30:0.5); C:DOPE-n-glutarylDOPE:PEG (69.5:30:0.5); D: DPPC:Chol:DODAC (30:40:30); E:DOPE:DOPS:PEG (69.5:30:0.5).

FIGURE 5. Killing of intracellular *Listeria monocytogenes* by liposomal gentamicin. J774 cells were treated as stated in Figure 4. A:DOPE:n-succinylDOPE (70:30); B:DOPE:n-succinylDOPE:PEG (69.5:30:0.5).

FIGURE 6. Influence of externally-bound gentamicin on antibacterial efficacy of liposomal formulations. J774 cells were treated as stated in Figure 4, except the gentamicin dose was 150 μg/mL. Free: unencapsulated gentamicin; ext: DOPE:n-succinyl DOPE:PEG (69.5:30:0.5) liposomes with gentamicin adsorbed to the external surface; int: liposomes of the same composition but with gentamicin internally encapsulated; ext + int: liposomes of the same composition with gentamicin both internal to the liposome and adsorbed to the surface.

FIGURE 7. Effect of inhibition of endosomal acidification on killing of intracellular recombinant *S. typhimurium* by liposomal gentamicin. J774 cells were treated as in Figure 4, except bafilomicin was added to some samples to 100 μg/mL. A: DOPE:n-succinylDOPE (70:30); B:DOPC:n-succinylDOPE(70:30).

FIGURE 8. Fusion activity of liposomes containing gentamicin at various pH. Fusion activity of gentamicin-containing DOPE:n-succinylDOPE:PEG (79.5:30:0.5) or DOPC:n-succinylDPPE:PEG (69.5:30:0.5) was monitored using a resonance energy transfer assay as described in Example 3. A = 1 min, B = 2 min, C = 5 min, D = 10 min.

FIGURE 9. Flow cytometry of fluorescent liposomal formulations incubated with COS cells. COS cells were incubated with liposomes as described in Example 4. Panel A indicates calcein fluorescence in COS cells. Solid line = no liposome. Dashed line = DOPE:n-succinylDOPE:PEG (69.5:30:0.5). Dotted line = DOPC:n-succinylDOPE:PEG (69.5:30:0.5). Panel B shows the fluorescence of the NBD-PE tag in the lipid carrier under identical circumstances to Panel A (same key applies). Panel C shows fluorescence of 5-sulfofluorescein diacetate delivered to COS cells by liposomes (same key

as panel A). Panel D shows the fluorescence of the NBD-PE tag in the lipid carrier under identical circumstances to Panel C (same key applies).

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

The present invention provides a novel combination of cationic drugs encapsulated in non-cationic liposomes. In a preferred embodiment, the liposomes are fusogenic, *i.e.*, upon a change in ambient conditions, such as pH, the lipid bilayer degrades or destabilizes and the drug is released. In a particularly preferred embodiment, the liposome degrades in the acidic environment of endosomal, lysosomal and/or phagocytic vesicles.

II. Definitions

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein. Units, prefixes, and symbols can be denoted in their SI accepted form. Numeric ranges are inclusive of the numbers defining the range. The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

The phrase "cationic drug" refers to a therapeutic compound having a net positive charge. The term "therapeutic compound" refers to a composition of matter which confers a therapeutic benefit to an organism with a pathological condition. The phrase "pathological condition" refers to a physiological state that normally does not exist, *i.e.*, a disease. In the context of the present invention, pathological conditions are caused by bacteria, *e.g.*, *Listeria*; fungi; viruses, *e.g.*, HIV; genetic disorders, *e.g.*, sickle cell anemia; immunodeficiency, *e.g.*,severe combined immunodeficiency (SCID) and irradiation; inflammation caused by infection and/or injury; and cancer, *e.g.*, leukemias and lymphomas. The term "ameliorate" refers to improving or treating a pathological condition. The improvement can be a cure for the pathological condition, a lessening of undesired physiological characteristics of the pathological condition, or an increase in the quality of life of a patient with the pathological condition.

The phrase "bacterial infection" refers to a pathological condition caused by bacteria. The infection can be systemic, *e.g.*, toxic shock, or localized to tissues, organs or cells. An "intracellular bacterial infection" is a bacterial infection resident within a cell. In this invention, many intracellular bacterial infections can be treated. These include, but are not limited to, *Listeria*, *Mycobacteria*, *Salmonella*, *Shigella*, *E. coli*, *Neisseria*, *Yersinia*, and *Legionella*.

The term "antibiotic" is a therapeutic compound in which the therapeutic effect after administration is the killing of pathogens including, but not limited to, bacteria, yeast and viruses. A preferred antibiotic of this invention is gentamicin. Gentamicin is a polycationic aminoglycoside produced by *Micromonospora purpurea*. Gentamicin is the generic name for 3 different but related compounds: "gentamicin C_1, gentamicin C_2, and gentamicin C_{1a}. Commercially supplied gentamicin is typically a mixture of the sulfate salts of the three compounds.

The term "liposome" refers to vesicles comprised of one or more concentrically ordered lipid bilayers which encapsulate an aqueous phase. Normally, the encapsulated matrix does not permeate the bilayer. However, if a hole or pore occurs in the bilayer, if the bilayer is dissolved or degraded, if the bilayer changes conformation, or if the environmental temperature is increased to the phase transition temperature, T_c, of the constituent lipids, the matrix may leak through the liposome.

The "interior" of a liposome is the aqueous area surrounded by the lipid bilayer of the liposome, *i.e.*, encapsulated matrix. The process of placing a compound within the aqueous matrix is termed "encapsulating." The "surface" of a liposome is the hydrophilic portion of the substituent lipids exposed to the extraliposomal environment. Binding of a compound to the surface of a liposome can be due to covalent bonding of the compound to the hydrophilic group, hydrogen bonding, electrostatic interactions, and hydrophobic/hydrophilic interactions. In addition to binding to the surface, a compound with a hydrophobic component can insert into the liposome bilayer so that the hydrophobic component is within the bilayer and the hydrophilic portion of the compound extends beyond the surface of the liposome.

The phrase "non-cationic liposome" refers to neutral and anionic liposomes. These are liposomes in which net charge of the hydrophilic groups is either neutral or negative at physiological pH, *i.e.*, pH 7.0 to 7.5.

The term "lipid" refers to any suitable material capable of forming a bilayer such that a hydrophobic portion of the lipid material orients toward the bilayer while a hydrophilic portion orients toward the aqueous phase. Amphipathic lipids are necessary as the primary lipid vesicle structural element. Hydrophilic characteristics derive from the presence of phosphato, carboxylic, sulfato, amino, sulfhydryl, nitro, and other like groups. Hydrophobicity can be conferred by inclusion of groups that include, but are not limited to, long chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group(s). The preferred amphipathic compounds are phosphoglycerides and sphingolipids, representative examples of which include phosphatidylcholine, phosphatidylethanolamine and its derivatives such as dioloylphosphatidylethanolamine (DOPE), phosphatidylglycerol, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid and glycosphingolipid families are also within the group designated as lipid. Additionally, the amphipathic lipids described above may be mixed with other lipids including triglycerides and sterols, such as cholesterol.

The term "neutral lipid" refers to any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides.

The term "non-cationic lipid" refers to any neutral lipid as described above as well as anionic lipids. Examples of anionic lipids include, but are not limited to, phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, n-dodecanoyl phosphatidylethanolamines, n-succinyl phosphatidylethanolamines, n-glutaryl phosphatidylethanolamines, lysylphosphatidylglycerols, and other anionic modifying groups joined to neutral lipids.

The term "acyl" refers to a radical produced from an organic acid by removal of the hydroxyl group. Examples of acyl radicals include acetyl, pentanoyl, palmitoyl, stearoyl, myristoyl, caproyl and oleoyl. The phrase "acyl chain" refers to the above acyl compounds which are joined to the hydrophilic head group of a lipid via reaction of the radical in the acyl.

The phrase "lipid derivative" refers to a lipid-based compound in which a functional group has been joined to the hydrophilic head. The functional group can be, for example, a space filling compound or a compound which binds specifically to another compound or cellular receptor. A preferred lipid derivative of this invention is PEG_n-lipid derivative. A most preferred lipid derivative is PEG_n-ceramide.

The term "fusogenic" refers to the ability of a liposome to fuse with membranes of an animal cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.* "Fusogenesis" is the fusion of a liposome to such a membrane. A fusogenic liposome comprises a lipid which assumes a bilayer structure, yet is capable of adopting a non-lamellar phase that fuses with membranes.

Lipids which can be used in the present invention to form fusogenic liposomes are those which adopt a non-lamellar phase under specific physiological conditions, *e.g.*, in the presence of calcium ions or in non-physiological pH, but which, in physiological conditions, assume a bilayer structure. Typically, to achieve a bilayer state, at least one bilayer stabilizing component is needed. Lipids which adopt a non-lamellar phase include, but are not limited to, phosphatidylethanolamines, ceramides, glycolipids, or mixtures thereof. Such lipids can be stabilized in a bilayer structure by bilayer stabilizing components which are either bilayer forming themselves, or which are of a complementary dynamic molecular shape. More particularly, the bilayer stabilizing components of the present invention must be capable of stabilizing the lipid in a bilayer structure, yet they must be capable of exchanging out of the liposome, of being chemically modified by endogenous systems or of being modified under changes in ambient conditions so that they lose their ability to stabilize the lipid in a bilayer structure, thereby allowing the liposome to become fusogenic. Only when liposomal stability is lost or decreased can fusion of the liposome with the membranes of the target cell occur.

The phrase "acidic environment" in the context of this invention refers to an intracellular organelle in which the pH of the interior of the organelle is less than about pH 7.0. Such organelles include, but are not limited to, endosomes, phagosomes, lysosomes, many types of vesicles (secretory and transport), and selected compartments of the Golgi apparatus.

The phrase "pharmaceutically acceptable carrier" refers to a solution which is physiologically compatible. Generally, normal saline will be employed as the

pharmaceutically acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* In compositions comprising saline or other salt containing carriers, the carrier is preferably added following particle formation. Thus, after the particle is formed, the particle can be diluted into pharmaceutically acceptable carriers such as normal saline. The carrier may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, *etc.* Additionally, the liposomal suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

III. Characterization and Formulation of Fusogenic Liposomes.

A. Fusogenic Liposomes

In one embodiment of the instant invention, the lipids comprising the liposome are vesicle-forming lipids. "Vesicle-forming lipid" is intended to include any amphipathic lipid having hydrophobic and polar head group moieties, and which (a) by itself can form spontaneously into bilayer vesicles in water, as exemplified by many of the phospholipids, or (b) is stably incorporated into lipid bilayers in combination with bilayer-forming phospholipids, with its hydrophobic moiety in contact with the interior, hydrophobic region of the bilayer membrane, and its polar head group moiety oriented toward the exterior, polar surface of the membrane. Examples of the latter type of vesicle-forming lipids are phosphatidylethanolamines, ceramides, glycolipids, cholesterol and cholesterol derivatives, such as cholesterol sulfate and cholesterol hemiscuccinate, and mixtures thereof.

In a preferred embodiment of the present invention, a fusogenic liposome is provided, the fusogenic liposome comprising: a lipid capable of adopting a non-lamellar phase under specific physiological conditions, yet assuming a bilayer structure under normal physiological conditions. In a particularly preferred embodiment, the lipids are stabilized in a bilayer structure by the presence of a bilayer stabilizing component.

Such fusogenic liposomes are advantageous because the rate at which they become fusogenic can be changed depending on the extraliposomal environment, *e.g.*, the

ion concentration or the pH of the environment. In particular, by varying the composition and concentration of the bilayer stabilizing component, one can control the rate at which the bilayer stabilizing component exchanges out of the liposome and, in turn, the rate at which the liposome becomes fusogenic. In addition, a bilayer stabilizing component within a lipid bilayer maintains the bilayer structure before administration of the liposome to the patient, thus extending the shelf-life of the liposome-encapsulated drug.

The polymorphic behavior of lipids in organized assemblies can be explained qualitatively in terms of the dynamic molecular shape concept (*see*, Cullis, *et al.*, in MEMBRANE FUSION, Wilschut, J. and Hoekstra, D. (eds.), Marcel Dekker, Inc., New York, (1991)). When the effective cross-sectional areas of the polar head group and the hydrophobic region buried within the membrane are similar, the lipids have a cylindrical shape and tend to adopt a bilayer conformation. Cone-shaped lipids which have polar head groups that are small relative to the hydrophobic component, such as unsaturated phosphatidylethanolamines, prefer non-bilayer phases such as inverted micelles or inverse hexagonal phase (H_{II}). Lipids with head groups that are large relative to their hydrophobic domain, such as lysophospholipids, have an inverted cone shape and tend to form micelles in aqueous solution. The phase preference of a mixed lipid system depends, therefore, on the contributions of all the components to the net dynamic molecular shape. As such, a combination of cone-shaped and inverted cone-shaped lipids can adopt a bilayer conformation under conditions where either lipid alone cannot (*see*, Madden & Cullis, *Biochim. Biophys. Acta* **684**:149-153 (1982)).

A more formalized model is based on the intrinsic curvature hypothesis (*see*, *e.g.*, Kirk, *et al.*, *Biochemistry* **23**:1093-1102 (1984)). This model explains phospholipid polymorphism in terms of two opposing forces. The natural tendency of a lipid monolayer to curl and adopt its intrinsic or equilibrium radius of curvature (R_o) which results in an elastically relaxed monolayer is opposed by the hydrocarbon packing constraints that result from the adoption of R_o. Factors that decrease the intrinsic radius of curvature, such as increased volume occupied by the hydrocarbon chains when double bonds are introduced, tend to promote H_{II} phase formation. Conversely, an increase in the size of the head group increases R_o and promotes bilayer formation or stabilization. Introduction of apolar lipids that can fill the voids between inverted lipid cylinders also promotes H_{II} phase formation (*see*, Gruner, *et al.*, *Proc. Natl. Acad. Sci. USA* **82**:3665-3669 (1989); and Sjoland, *et al.*, *Biochemistry* **28**:1323-1329 (1989)).

Lipids which can be used to form the fusogenic liposomes of the present invention are those which adopt a non-lamellar phase under specific physiological conditions, *e.g.*, in the presence of calcium ions or in an acidic environment, but which are capable of assuming a bilayer structure under general physiological conditions. Such lipids include, but are not limited to, phosphatidylethanolamines, ceramides, glycolipids, or mixtures thereof. Other lipids known to those of skill in the art to adopt a non-lamellar phase under physiological conditions can also be used.

In a presently preferred embodiment, the fusogenic liposome is prepared from a phosphatidylethanolamine. Phosphatidylethanolamines have a variety of acyl chain groups of varying chain lengths and degrees of saturation. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skill in the art.

Phosphatidylethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, the following: dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphatidylethanolamine (DSPE).

In an equally preferred embodiment, the fusogenic liposome is prepared from a mixture of a phosphatidylethanolamine (saturated or unsaturated) and a phosphatidylcholine. A particularly preferred phosphatidylcholine is dioleoylphosphatidylcholine (DOPC). In another equally preferred embodiment, the fusogenic liposome is prepared from a mixture of a neutral phosphatidylethanolamine (saturated or unsaturated) and an anionic lipid. Because of the polar head group, in an altered pH environment, the net charge on the head group changes and the anionic lipid will exchange out of the liposome bilayer. In this way, does the liposome become fusogenic.

Examples of suitable anionic lipids include, but are not limited to, the following: phosphatidylserine, n-dodecanoyl phosphatidylethanolamines, n-succinyl phosphatidylethanolamines, n-glutaryl phosphatidylethanolamines, lysylphosphatidylglycerols, and other anionic modifying groups joined to neutral lipids.

In the instant invention, the anionic lipid also serves as a lipid bilayer stabilizing component. A bilayer stabilizing component is either bilayer forming itself, or is

of a complementary dynamic shape. In selecting an appropriate bilayer stabilizing component, it is imperative that the bilayer stabilizing component be capable of transferring out of the liposome, such as an anionic lipid in an acidic environment, or of being chemically modified by endogenous systems such that, with time, it loses its ability to stabilize the lipid in a bilayer structure. Only when liposomal stability is lost or decreased can fusion of the liposome with the membranes of the target cell occur. The bilayer stabilizing component is, therefore, "reversibly associated" with the lipid and only when it is associated with the lipid does the lipid adopt the bilayer structure.

Examples of suitable bilayer stabilizing components include, but are not limited to, lipids, such as the anionic lipids of the instant invention; lipid-derivatives; detergents; proteins and peptides. In a presently preferred embodiment, the bilayer stabilizing component is selected from the group consisting of n-succinylDOPE and n-glutarylDOPE. In another preferred embodiment, the bilayer stabilizing component is polyethylene glycol conjugated to, *i.e.*, coupled to, a phosphatidylethanolamine. In an equally preferred embodiment, the bilayer stabilizing component is polyethylene glycol conjugated to a ceramide.

Polyethylene glycol can be conjugated to a phosphatidylethanolamine or, alternatively, to a ceramide using standard coupling reactions known to and used by those of skill in the art. In addition, preformed polyethylene glycol-phosphatidylethanolamine conjugates are commercially available from Avanti Polar Lipids (Alabaster, Alabama).

Polyethylene glycols of varying molecular weights can be used to form the bilayer stabilizing components of the present invention. Polyethylene glycols of varying number of ethylene glycol units (and therefore molecular weight) are commercially available from a number of different sources or, alternatively, they can be synthesized using standard polymerization techniques well-known to those of skill in the art. In a presently preferred embodiment, the polyethylene glycol has from about 10 to about 30 ethylene glycol units. In a particularly preferred embodiment, the polyethylene glycol has from about 10 to 20 ethylene glycol units. In a most preferred embodiment, the polyethylene glycol has from about 14 to about 20 ethylene glycol units.

Generally, it has been found that increasing the number of ethylene glycol units (and thus the molecular weight) of the polyethylene glycol reduces the concentration of the bilayer stabilizing component required to achieve stabilization.

As with the phosphatidylethanolamines described above, ceramides having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be coupled to polyethylene glycol to form the bilayer stabilizing component. It will be apparent to those of skill in the art that in contrast to the phosphatidylethanolamines, ceramides have only one acyl group which can be readily varied in terms of its chain length and degree of saturation. Ceramides suitable for use in accordance with the present invention are commercially available. In addition, ceramides can be isolated, for example, from egg or brain using well-known isolation techniques or, alternatively, they can be synthesized using the methods and techniques disclosed in U.S. Patent Application Serial Number 08/316,429, filed September 30, 1994, and U.S. Patent Application Serial No. 08/486,214, filed June 7, 1995, the teachings of which are incorporated herein by reference. Using the synthetic routes set forth in the foregoing application, ceramides having saturated or unsaturated fatty acids with carbon chain lengths in the range of C_2 to C_{31} can be prepared. Particularly preferred ceramides have an acyl chain length of from about 10 to about 25, with the most preferred ceramide having an acyl chain length of about 20 carbon atoms.

By controlling the composition and concentration of the bilayer stabilizing component, one can control the rate at which the liposome becomes fusogenic. For instance, when a polyethylene glycol-phosphatidylethanolamine conjugate or a polyethylene glycol-ceramide conjugate is used as the bilayer stabilizing component, the rate at which the liposome becomes fusogenic can be varied, for example, by varying the concentration of the PEG-lipid derivative, by varying the molecular weight of the polyethylene glycol, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the liposome becomes fusogenic. Other methods which can be used to control the rate at which the liposome becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

In addition to its activity as a bilayer stabilizing component, PEG-lipid derivatives also serve to prevent aggregation of the liposome during its formation and storage. Thus, PEG-lipid derivatives, such as PEG-ceramide, is a formulation agent.

In a presently preferred embodiment, the fusogenic liposomes contain cholesterol. It has been determined that when cholesterol-free liposomes are used *in vivo*, they have a tendency to absorb cholesterol from plasma lipoproteins and cell membranes.

Since this absorption of cholesterol could, in theory, change the fusogenic behavior of the liposomes, cholesterol can be included in the fusogenic liposomes of the present invention so that little or no net transfer of cholesterol occurs *in vivo*. Cholesterol, if included, is generally present at a concentration ranging from 0.02 mole percent to about 50 mole percent and, more preferably, at a concentration ranging from about 35 mole percent to about 45 mole percent.

B. Formulation of Fusogenic Liposomes

A variety of methods are available for preparing liposomes as described in, *e.g.*, Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* **9**:467 (1980), U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, 4,946,787, PCT Publication No. WO 91\17424, Deamer & Bangham, *Biochim. Biophys. Acta* **443**:629-634 (1976); Fraley, *et al.*, *Proc. Natl. Acad. Sci.* USA **76**:3348-3352 (1979); Hope, *et al.*, *Biochim. Biophys. Acta* **812**:55-65 (1985); Mayer, *et al.*, *Biochim. Biophys. Acta* **858**:161-168 (1986); Williams, *et al.*, *Proc. Natl. Acad. Sci. USA* **85**:242-246 (1988); the text LIPOSOMES, (Marc J. Ostro (ed.), Marcel Dekker, Inc., New York, 1983, Chapter 1); and Hope, *et al.*, *Chem. Phys. Lip.* **40**:89 (1986), all of which are incorporated herein by reference. Suitable methods include, for example, sonication, extrusion, high pressure/homogenization, microfluidization, detergent dialysis, calcium-induced fusion of small liposome vesicles and ether-fusion methods, all of which are well known in the art. One such method produces multilamellar vesicles of heterogeneous sizes. In this method, the vesicle-forming lipids are dissolved in a suitable organic solvent or solvent system and dried under vacuum or an inert gas to form a thin lipid film. If desired, the film may be redissolved in a suitable solvent, such as tertiary butanol, and then lyophilized to form a more homogeneous lipid mixture which is in a more easily hydrated powder-like form. This film is covered with an aqueous buffered solution and allowed to hydrate, typically over a 15-60 minute period with agitation.

In a preferred embodiment, the relative amounts of phosphatidylethanolamine and anionic lipid are about 70:30. However, one of skill will appreciate that the ratio can range from 33:67 to about 90:10. In a particularly preferred embodiment, PEG_n-lipid derivative is added to the mixture of lipids at a relative molar concentration of about 0.25 to 20%. In one preferred embodiment, the relative molar concentration is 0.5. In an *in vivo* use of the liposomes of the instant invention, 5.0% is most preferred.

In a preferred method for making the liposomes of this invention, the appropriate lipids are solubilized in a nonpolar solvent, such as chloroform. The solvent is evaporated according to well-known methods, for example, under a stream of nitrogen gas. Optimally, the lipids are then dried to eliminate all solvent (both polar solvents and any atmospheric water that may have contaminated the solvent). The dried lipid film is then rehydrated in an aqueous solution containing the cationic drugs of this invention (*see infra*). Because the drug is cationic, it is important to include buffering salts to the aqueous solution to maintain a pH in which bilayer structures can form. Such buffering salts include, but are not limited to, HEPES, sodium and potassium phosphates, citrate and Tris. In addition to the buffering salts, one of skill will appreciate that other salts can be added to the aqueous solution. These salts include, but are not limited to, sodium chloride, potassium chloride, sodium acetate, sodium lactate, calcium chloride, and the like.

In addition to the method described above, other methods of loading cationic drugs into non-cationic fusogenic liposomes include encapsulation and transmembrane potential loading. In one encapsulation technique, the drug and liposome components are dissolved in an organic solvent in which all species are miscible and concentrated to a dry film. A buffer is then added to the dried film and liposomes are formed having the drug incorporated into the vesicle walls. In this manner, the drug will become encapsulated in the aqueous interior of the liposome. The buffer which is used in the formation of the liposomes can be any biologically compatible buffer solution of, for example, isotonic saline, phosphate buffered saline, or other low ionic strength buffers. The resulting liposomes with the drug incorporated in the aqueous interior or in the membrane are then optionally sized as described above.

Transmembrane potential loading has been described in detail in U.S. Patent No. 4,885,172, U.S. Patent No. 5,059,421, and U.S. Patent No. 5,171,578, the contents of which are incorporated herein by reference. Briefly, the transmembrane potential loading method can be used with essentially any conventional drug which exhibits weak acid or weak base characteristics. Preferably, the drug will be relatively lipophilic so that it will partition into the liposome membrane. A pH gradient is created across the bilayers of the liposomes or protein-liposome complexes, and the drug is loaded into the liposome in response to the pH gradient. The pH gradient is generated by creating a proton gradient across the membrane either by making the interior more acidic or basic than the exterior (Harrigan, *et al.*, *Biochem. Biophys. Acta.* **1149**:329-339 (1993), the teachings of which are

incorporated herein by reference), or by establishing an ion gradient employing ionizable agents, such as ammonium salts, which leads to the generation of a pH gradient (*See*, U.S. Patent No. 5,316,771) the teachings of which are incorporated herein by reference).

The size distribution of the resulting multilamellar vesicles can be shifted toward smaller sizes by hydrating the lipids under more vigorous agitation conditions or by adding solubilizing detergents such as deoxycholate.

Unilamellar vesicles are generally prepared by sonication or extrusion. Sonication is generally preformed with a tip sonifier, such as a Branson tip sonifier, in an ice bath. Typically, the suspension is subjected to several sonication cycles. Extrusion can be carried out by biomembrane extruders, such as the Lipex Biomembrane Extruder. Defined pore size in the extrusion filters can generate unilamellar liposomal vesicles of specific sizes. The liposomes can also be formed by extrusion through an asymmetric ceramic filter, such as a Ceraflow Microfilter, commercially available from the Norton Company, Worcester MA. In a preferred embodiment, the lipid/drug mixture is repeatedly extruded, above the T_c, through a biomembrane filter. In a particularly preferred embodiment, the membrane has a pore size of about 100 nm and extrusion is repeated about 10 times. If necessary, the lipid/drug mixture can be initially passed through a larger pore size membrane, for example 200 nm.

Following liposome preparation, the liposomes may be sized to achieve a relatively narrow distribution of desired liposome sizes. A size range of about 0.05 μm to about 0.20 μm allows the liposome suspension to be sterilized by filtration through a conventional filter, typically a 0.22 μm filter. The filter sterilization method can be carried out on a high throughput basis if the liposomes have been sized down to about 0.05 μm to about 0.20 μm.

Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles which are less than about 0.05 μm in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 μm, are observed. In both of these methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination.

In addition, the size of the liposomal vesicle can be determined by quasi-electric light scattering (QELS) as described in Bloomfield, *Ann. Rev. Biophys. Bioeng.* 10:421-450 (1981), incorporated herein by reference. Average liposome diameter can be reduced by sonication of formed liposomes. Intermittent sonication cycles can be alternated with QELS assessment to guide efficient liposome synthesis.

Extrusion of liposomes through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing liposome sizes to a relatively well-defined distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. For use in the present inventions, liposomes having a size of from about 0.05 μm to about 0.45 μm are preferred.

In certain embodiments of the present invention, it is desirable to target the liposomes of the invention using targeting moieties that are specific to a particular cell type, tissue, and the like. Targeting of liposomes using a variety of targeting moieties (*e.g.*, ligands, receptors and monoclonal antibodies) has been previously described (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044, both of which are incorporated herein by reference).

Examples of targeting moieties include monoclonal antibodies specific to antigens associated with neoplasms, such as prostate cancer specific antigen. Tumors can also be diagnosed by detecting gene products resulting from the activation or overexpression of oncogenes, such as ras or c-erbB2. In addition, many tumors express antigens normally expressed by fetal tissue, such as the alphafetoprotein (AFP) and carcinoembryonic antigen (CEA). Sites of viral infection can be diagnosed using various viral antigens such as hepatitis B core and surface antigens (HBVc, HBVs) hepatitis C antigens, Epstein-Barr virus antigens, human immunodeficiency type-1 virus (HIV1) and papilloma virus antigens. Inflammation can be detected using molecules specifically recognized by surface molecules which are expressed at sites of inflammation such as integrins (*e.g.*, VCAM-1), selectin receptors (*e.g.*, ELAM-1) and the like.

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes lipid components, *e.g.*, phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid derivatized bleomycin. Antibody targeted liposomes can be constructed using, for instance, liposomes which incorporate protein A

(*see*, Renneisen, *et al.*, *J. Biol. Chem.*, **265**:16337-16342 (1990) and Leonetti, *et al.*, *Proc. Natl. Acad. Sci. (USA)* **87**:2448-2451 (1990), both of which are incorporated herein by reference).

Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The liposome is typically fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion which is firmly embedded and anchored in the membrane. It must also have a hydrophilic portion which is chemically available on the aqueous surface of the liposome. The hydrophilic portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent which is added later. Therefore, the connector molecule must have both a lipophilic anchor and a hydrophilic reactive group suitable for reacting with the target agent and holding the target agent in its correct position, extended out from the liposome's surface. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is in the membrane with the target agent which is extended, three dimensionally, off of the vesicle surface.

IV. Cationic Drugs

Any variety of cationic drugs which are selected as appropriate for the intracellular disease to be treated can be administered using the fusogenic liposomes of the present invention. Often the drug will be an antineoplastic agent, such as aminoglutethimide, cytarabine, dacarbazine, dactinomycin, procarbazine, and the like. It may also be desirable to deliver anti-infective agents to specific cells by the present methods. Anti-infectives include anti-bacterial, anti-viral, anti-fungal and anti-parasitic agents. These compositions include, but are not limited to, aminoglycosides, such as gentamicin, kanamycin, neomycin, streptomycin and tobramycin; cephalosporins, such as ceclor, defadroxil, claforan, mefoxin and the like; penicillins, such as amoxicillin, ampicillin and penicillin; tetracyclines, such as declomycin, vibramycin, meclan, minocyn, terramycin, and tetracycline; and other anti-infectives, including chloramphenicol, isoniazid, trimethoprim and small cationic peptides or large peptides with polycationic domains such as protegrin and indolicidin. The liposomes of the present invention can also be used for the selective

delivery of other cationic drugs including, but not limited to, local anesthetics, beta-adrenergic blockers, antihypertensive agents, anti-depressants, anti-convulsants, antihistamines, hormones, hormone antagonists, immunomodulators, neurotransmitter antagonists, vitamins, narcotics, and imaging agents. Other particular drugs which can be selectively administered by the compositions of the present invention will be well known to those of skill in the art. Additionally, two or more cationic drugs can be administered simultaneously if desired, where such drugs produce complementary or synergistic effects.

In a preferred embodiment, the cationic drug is an antibiotic and, in a particularly preferred embodiment, the antibiotic is gentamicin. To load gentamicin and/or other cationic drugs into the liposomes, the lipid constituents of the liposome are rehydrated in an aqueous solution containing the cationic drug(s). The concentration of the drug(s) in the aqueous solution depends, in addition to other factors, on the solubility of the drug(s) in aqueous solutions and the amount of drug(s) to be delivered. The amount to be delivered depends on the type of drug, *e.g.*, anti-microbial, antineoplastic, *etc.*, and the toxicity of the drug. Typically, the concentration of cationic drug in the rehydration solution is from about 0.1 ng/mL to 150 mg/mL. In a particularly preferred embodiment of the invention, gentamicin is added to 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) at a concentration of 50 to 150 mg/mL.

Because the drugs of this invention are cationic and the liposomes may be anionic, encapsulation of the drug within the liposome is improved over other drug/liposome compositions. However, these same properties may allow the binding of the drug to the surface of the liposome through electrostatic interactions. In a preferred embodiment, the drug bound to the external surface of the liposome is removed by a variety of means, but a preferred method is to adjust the pH of the solution containing the liposomes to pH 9-12 with a gentle base solution, such as glycine buffered saline. The liposomes are then passed over a CM-SEPHAROSE (Amersham, Cambridge, MA) column equilibrated with a buffer of the same pH as the liposome solution. The ionic strength of the resin is greater than that of the liposome and the cationic drug preferentially binds to the column resin. The net positive charge on the liposomes causes the liposomes to pass through the column. The column flow-through, after collection, is brought to a physiological pH by any number of means known to those of skill, including, but not limited to, dilution, diafiltration, or dialysis.

V. Pharmaceutically Acceptable Carriers

Following a separation step as may be necessary to remove free drug from the medium containing the liposome or drug that is bound to the external surface of the liposome, the liposome suspension is brought to a desired concentration in a pharmaceutically acceptable carrier for administration to the patient or host cells. The dosage of the pharmaceutical composition can be determined by *in vivo* assays and dosage scaling which are known to those skilled in the art. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found in REMINGTON'S PHARMACEUTICAL SCIENCES, 17TH ED., Mack Publishing Company, Philadelphia, PA, (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

The concentration of liposomes in the carrier may vary. Generally, the concentration will be about 10-200 mg/mL, usually about 10-150 mg/mL, and most usually about 25 mg/mL. Persons of skill may vary these concentrations to optimize treatment with different liposome components or for particular patients. For example, the concentration may be increased to lower the fluid load associated with treatment.

The present invention also provides methods for introducing cationic drugs into cells of a host. The methods generally comprise administering to the host a fusogenic liposome containing the cationic drug, wherein the fusogenic liposome comprises a bilayer stabilizing component and/or a non-cationic lipid which adopts a bilayer structure under

physiological conditions, yet which is capable of assuming a non-lamellar phase in an acidic environment, for example, in the lysosome, phagosome and endosome of a cell. The host may be a variety of animals, including humans, non-human primates, avian species, equine species, bovine species, swine, lagomorpha, rodents, and the like.

The cells of the host are usually exposed to the liposomal preparations of the invention by *in vivo* administration of the formulations, but *ex vivo* exposure of the cells to the liposomes is also feasible. *In vivo* exposure is obtained by administration of the liposomes to host. The liposomes may be administered in many ways. These include parenteral routes of administration, such as intravenous, intramuscular, subcutaneous, and intraarterial. Generally, the liposomes will be administered intravenously or in some cases via inhalation. Often, the liposomes will be administered into a large central vein, such as the superior vena cava or inferior vena cava, to allow highly concentrated solutions to be administered into large volume and flow vessels. The liposomes may be administered intraarterially following vascular procedures to deliver a high concentration directly to an affected vessel. In some instances, the liposomes may be administered orally or transdermally, although the advantages of the present invention are best realized by parenteral administration. The liposomes may also be incorporated into implantable devices for long duration release following placement.

As described above, the liposomes will generally be administered intravenously or via inhalation in the methods of the present invention. Often multiple treatments will be given to the patient. The dosage schedule of the treatments will be determined by the disease and the patient's condition. Standard treatments with therapeutic compounds that are well known in the art may serve as a guide to treatment with liposomes containing the therapeutic compounds. The duration and schedule of treatments may be varied by methods well known to those of skill, but the increased circulation time and decreased liposomal leakage will generally allow the dosages to be adjusted downward from those previously employed. The dose of liposomes of the present invention may vary depending on the clinical condition and size of the animal or patient receiving treatment. The standard dose of the therapeutic compound when not encapsulated may serve as a guide to the dose of the liposome-encapsulated compound. The dose will typically be constant over the course of treatment, although in some cases the dose may vary. Standard physiological parameters may be assessed during treatment that may be used to alter the dose of the liposomes of the invention.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner.

VI. Examples

A. Example 1. Formulation of gentamicin-containing liposomes.

The formulations of liposomes are shown in Table 1. Appropriate amounts of lipids (Avanti polar lipids Inc. Alabaster, AL, USA or Northern Lipids, Vancouver, BC, Canada; PEG-C_{20}ceramide was from INEX, Vancouver, Canada) in molar ratios of DOPE:n-succinylDOPE:PEG-C_{20} ceramide (69.5:30:0.5) were solubilized to 25 mg/mL in chloroform and the chloroform was evaporated by agitation under a nitrogen stream. The lipid mixture was then dried under vacuum either overnight or for one hour to a film. Dried lipid films were rehydrated in gentamicin sulfate (Sigma, St. Louis, MO) in HBS (HEPES Buffered Saline; 20 mM HEPES, 150 mM NaCl, pH 7.4) at various concentrations (30 - 150 mg/mL gentamicin base) and subjected to five cycles of freezing in liquid nitrogen for approximately 5 min. and thawing at 30°C. The lipid/drug mixture was then extruded at 25-28°C through 100 nm filters ten times. Depending on the formulation, five passes through 200 nm filters were sometimes performed initially. Gentamicin bound to the surface of anionic liposomes was removed by column chromatography (see below). Lipid concentration was calculated either by addition of 5-10 μCi of ^{3}H or ^{14}C-cholesterol hexadecyl ether (Amersham, Cambridge, MA) and scintillation counting, or by phosphate determination by the method of Fiske and Subbarow (*see*, Leloir, L. & Cardini, C., METHODS OF ENZYMOLOGY, VOL. III, Colowick & Kaplan (eds.), pp840-50, Academic Press, N.Y., 1957). Gentamicin levels were determined either by fluorescence polarization immunoassay (FPIA), or by the addition of a known quantity of ^{3}H-gentamicin (Amersham) to the gentamicin stock solution, followed by scintillation counting.

Liposome sizes were routinely determined by quasielastic light scattering using a NICOMP 7600 submicron particle sizer.

Table 1. Liposomal Gentamicin Formulations

Formulation (Molar Ratio)	Typical Drug/Lipid Ratio (g/g)*
DPPC:Chol(70:30)	0.075
DPPC:Chol:DODAC(30:40:30)	0.16
DOPE:DOPS:PEG(69.5:30:0.5)	0.08
DOPE:n-succinylDOPE(70:30)	0.15
DOPE:n-succinylDOPE:PEG(69.5:30:0.5)	0.14
DOPE:n-succinylDOPE:PEG(69.5:30:1.0)	0.21
DOPE:n-glutarylDOPE(70:30)	0.15
DOPE:n-glutarylDOPE:PEG(69.5:30:0.5)	0.12
DOPC:n-succinylDOPE(70:30)	0.16
DOPC:n-succinylDOPE:PEG(69.5:30:0.5)	0.19

* with initial total lipid concentration of 25 mg/mL and an initial gentamicin base concentration of 64.5 mg/mL

DPPC = dipalmitoylphosphatidylcholine

Chol = cholesterol

DODAC = dioleoyldimethyl ammonium chloride

n-succinylDOPE = n-succinyldioleoylphosphatidylethanolamine

PEG = polyethylene glycol-ceramide (C20)

DOPE = dioleoylphosphatidylethanomine

n-glutarylDOPE = n-glutaryldioleoylphosphatidylethanolamine

DOPC = dioleoylphosphatidylcholine

Removal of gentamicin externally bound to anionic liposomes. Since gentamicin is a cationic molecule, it was probable that a proportion of the drug was bound by electrostatic interactions to the external surface of anionic liposomes. To determine the quantity of bound gentamicin, gentamicin (including ^{3}H-gentamicin) was incubated with empty liposomes for 30 min. at room temperature at a drug to lipid ratio of 2 to 1. The liposomes were dialyzed overnight against HBS and the gentamicin content measured as described above. The drug-to-lipid ratio was determined and compared to an identical lipid film rehydrated and extruded in gentamicin solution.

Removal of gentamicin from the surface of anionic liposomes was performed by adjustment of the pH of the liposome suspension to pH 10 with a small quantity of glycine buffered saline (1 M glycine, 150 mM NaCl, pH 10.0). The liposomes were then passed through a 20-25 mL CM SEPHAROSE (Amersham) column equilibrated to pH 10.0 with 10 mM glycine, 150 mM NaCl. The liposome-containing void volume was collected and re-equilibrated to pH 7.4 with 0.5 M HEPES, 150 mM NaCl pH 7.4. For DODAC and rhodamine-containing formulations, purification was by overnight dialysis in HBS followed by passage through a SEPHADEX G 50 (Amersham) column equilibrated to pH 10 as above. The liposomes were subsequently readjusted to pH 7.4 as above.

Figure 1 shows the amount of external gentamicin bound to the anionic formulation. Approximately 25 to 33% of total gentamicin appears to be associated with the outer surface when estimated by this method.

Drug retention. Retention of gentamicin in liposomes was determined by dialysis (12-14,000 Da cutoff) of radioactive liposome samples against either HBS or Citric Acid Buffer, pH 4.5 (CAB). HBS pH 7.4 was used as it is the standard storage buffer for these liposomes. CAB pH 4.5 was used to simulate the low pH of the phagosomes upon acidification. Dialysis took place at 37 °C with 100 µL samples taken at time points (to 24 h) and passed through 1 mL SEPHADEX G 50 spin columns (to remove gentamicin external to the liposomes, but inside the dialysis bag) before scintillation counting. Drug-to-lipid ratios for each time point were calculated, and retention expressed as the percent drug-to-lipid ratio at any one time point relative to that for the initial time point. See Figure 2.

The properties of the DOPE:n-succinylDOPE formulation had been previously investigated by Nayar and Schroit (Nayar, R. & Shroit, A., *Biochemistry* **24**:5967-5971 (1985)). These investigators found that 7:3 DOPE:n-succinylDOPE vesicles were more leaky under acidic conditions than at neutral pH (using an ANTS/DPX leakage assay, which detects a non-therapeutic fluorophore), which they hypothesized may be due to lipid packing defects that arise when the n-succinylDOPE is protonated. However, in the present example, the DOPE:n-succinyl DOPE formulation showed surprisingly little leakage in HBS or in CAB over 24 h at 37°C. The DPPC-based formulation showed no leakage under any of the conditions. These results show that the liposomes of the instant invention demonstrate a surprising ability to take up cationic drugs at high concentration.

At the above drug and lipid concentrations, anionic lipids aggregated, leading to significant loss of drug and lipid during the extrusion process (aggregates remaining on the filter). This effect was especially pronounced for DOPE:N-succinyl DOPE (70:30 mol:mol), where lipid loss during the filtration process was greater than 90%. To alleviate this problem, small amounts of PEG-C_{14}ceramide were added to the formulation. Post-extrusion yields of DOPE:n-succinyl DOPE:PEG (69.5:30:0.5 mol:mol) were routinely greater than 80%.

Quasielastic light scattering size data for these formulations showed the expected size distribution around a mean of approximately 100 nm.

B. Example 2: Uptake of liposomal gentamicin into J774 cells and intracellular killing.

Uptake. Since the ability of the liposomes to be taken up by cells is an important initial step in delivery of a drug, the uptake of the liposome formulations to J774 cells was assessed. The mouse macrophage-like cell line J774A1 (American Type Culture Collection, Rockville, MD) was used to study the attachment and uptake properties of the various liposome formulations. Cells were seeded at $5x10^5$ cells/well in 24- well plates with Dulbecco's modified Eagle's medium (DMEM, Gibco; Gaithersburg, MD) containing 10% v/v fetal bovine serum (FBS; Flow Laboratories) and grown overnight. The next day, cells were washed 3x in phosphate buffered saline plus Ca^{2+} and Mg^{2+}(PBS++; Gibco), Radiolabeled liposomes (200 µL) were added to a lipid concentration of 0.5 or 1 µmol/mL in growth medium. The cells were incubated with the liposomes for 2 h at 37°C, 5% CO_2, then washed 3x in PBS++. Cells were solubilized in 1% TRITON X-100 in 0.1% SDS (in PBS++) for 5 min. The cell-associated radioactivity was determined by liquid scintillation counting of the lysate. In order to differentiate active uptake from passive binding to the cells, uptake was routinely performed at both 37°C and 4°C. Cell-associated radioactivity at 4°C was assumed to be entirely due to passive binding, whereas at 37°C it was assumed to be the sum of binding and uptake. Hence, uptake was determined by subtraction of the 4°C data from the 37°C data.

Figure 3 indicates the uptake characteristics of several formulations. The formulation that was taken up by J774 cells in the greatest quantities is the cationic DPPC:Chol:DODAC (3:4:3) formulation. This result was expected since the surface of cells

holds a net negative charge, so electrostatic attraction brought the two bodies together, facilitating uptake. The anionic analog of the above formulation, DPPC:Chol:DOPS (3:4:3:) was taken up poorly by the cells in comparison, which could be due to electrostatic repulsion. Surprisingly, electrostatic repulsion was not observed with the DOPE:n-succinyl DOPE formulations (C - F). Although negatively charged, they nevertheless show enhanced binding over the DOPS-containing formulation. The addition of PEG-C_{20} ceramide to these formulations, as expected, lowered the amount of passive binding. However, the effect was not evident until the PEG concentration was greater than 2%.

Killing of intracellular bacteria. To test for efficacy of the liposomal formulations against intracellular infections, a tissue culture assay was developed where cells were first infected with intracellular-residing bacteria, then exposed to external gentamicin formulations.

The bacteria used in these experiments were either wild-type *Salmonella typhimurium* SL1344, recombinant *S. typhimurium* SL1344-HLY$^+$ expressing *E. coli* hemolysin, or wild type *Listeria monocytogenes* L028. *S. typhimurium* strains were cultured standing at 37°C in Luria-Bertrani (LB) broth overnight, while *L. monocytogenes* were grown in shaking cultures at 37°C in Brain Heart Infusion (BHI) broth also overnight, then subcultured for 2 h. All bacteria were stored on plates at 4°C or in frozen stocks at -70°C. Before cell infection, bacterial suspensions were washed once in PBS++ to remove accumulated hemolysin, then adjusted to an optical density (OD) of 0.5 at 600 nm. Cells, (J774, $5x10^5$ cells/well) were grown in 24-well plates overnight at 37°C in 5% CO_2 and DMEM-10% FBS. For *Salmonella* strains, the cells were infected at 5×10^5 cfu/10^6 cells for 15-20 min; for *Listeria* strains cells were infected at 10^6 cfu/10^6 cells for 30-40 min. All infections took place at 37°C and 5% CO_2.

After infection with the bacteria, the cells were washed three times with PBS++ then treated with gentamicin (100 µg/mL) for 1 h, under the same conditions. This was done to kill all bacteria that remained extracellular, leaving only the bacteria located inside the cells.

Cells were washed twice in PBS++ after gentamicin treatment, the liposome samples were added to the cells and the plates were incubated for a further 2 h under the same conditions. The control was free gentamicin at an equivalent concentration. Cells were dosed either by lipid concentration (1 µmol/mL) or by gentamicin concentration

(generally 150 μg/mL). Finally, cells were washed 3x with PBS++, then lysed in 1% TRITON X-100 in 0.1% SDS and dilutions of the lysate were plated on either LB plates (*S. typhimurium*) or tryptic soy agar (TSA) plates (*L. monocytogenes*). After overnight incubation at 37°C, colony forming units were counted and comparisons made between the controls (no liposome treatment or free gentamicin) and liposome treated wells.

Figure 4 shows the results of killing assays utilizing J774 cells infected with either wild-type *S. typhimurium* SL 1344 or recombinant *S. typhimurium* SL 1344 expressing the *E. coli* hemolysin. Initially, neutral, anionic and cationic DPPC-based liposomes were tested for anti-*Salmonella* activity. The neutral formulations showed no antibacterial activity. Anionic DPPC-based liposomes also showed no antibacterial activity. In fact, only DODAC-containing cationic liposomes, which were thought to have the greatest probability of bactericidal activity due to previous binding/uptake findings (Figure 3), showed activity against wild-type *Salmonella*, killing approximately 40% of intracellular bacteria (Figure 4A).

Anionic fusogenic liposomes then were tested for the ability to kill intracellular bacteria. DOPE:n-succinyl DOPE liposomes containing gentamicin at a drug-to-lipid ratio of 0.16 were found to be active against intracellular wild-type *S. typhimurium*, killing 76% of bacteria compared to no liposome treatment (Figure 4A). Since wild-type *S. typhimurium* resides within the phagosome during the assay, the gentamicin must have been released from the liposome in the endosome and then migrated to the phagosome.

To investigate the killing of intracellular bacteria not found in the phagosome, invasion assays were performed using a recombinant *Salmonella* strain that expressed an *E. coli* hemolysin. This bacterium was able to escape the phagosome by lysis of the phagosomal membrane by the secreted hemolysin protein. The bacteria then survived free in the cell cytosol. DOPE:n-succinyl DOPE liposomes containing gentamicin were very successful at killing these cytosol-residing intracellular bacteria. Figure 4B shows that 88% of recombinant *Salmonella* were killed compared to no treatment over the period of the assay (2 hours).

PEG-ceramide containing DOPE:n-succinyl DOPE and DOPE:n-glutaryl DOPE formulations were tested for the ability to kill intracellular bacteria. Figure 4 shows the effect of PEG-ceramide on the killing activity of gentamicin-filled DOPE-based liposomes. At a PEG-C_{20} ceramide concentration of 0.5 mol% there was no significant

effect on the antibacterial efficacy of these formulations against either wild-type or homolysin-expressing *S. typhimurium.*

Substitution of n-glutaryl DOPE for n-succinyl DOPE in liposomes also gave identical efficacy to the n-succinyl formulation against the test bacteria (Figure 4A and B). The killing effect was specific to n-succinyl and n-glutaryl-containing formulations as a formulation containing an equal molar proportion of a different anionic lipid, DOPS, showed very limited killing activity against recombinant *S. typhimurium.*

Liposomal gentamicin formulations were also tested against J774 cells infected with *Listeria monocytogenes. L. monocytogenes* naturally expresses a specialized hemolysin called listeriolysin. The listeriolysin molecule only becomes active when the pH of the endosome drops to approximately 5.5. At this pH, the endosomal membrane is lysed and the bacteria escapes to live free in the cytosol. Figure 5 shows that DOPE:n-succinyl DOPE liposomes (formulated with and without PEG ceramide) show near total killing of these bacteria. Greater killing of *L. monocytogenes* and the hemolysin-expressing *S. typhimurium* could be because the drug needed to only escape the endosome to encounter the bacteria in the cytosol (as opposed to the wild-type vacuole-bound *S. typhimurium).*

In a previous section, it was established that these gentamicin formulations possess a small proportion of the total gentamicin content bound to the exterior surface of the vesicle. Therefore, to test the influence of this externally bound gentamicin on the liposomes ability to kill bacteria, a killing assay was performed using J774 cells infected with hemolysin-expressing *S. typhimurium.* These cells were treated with DOPE:n-succinyl DOPE:PEG-ceramide liposomes with internal gentamicin only (externally bound drug removed by column chromatography) or with external gentamicin only (prepared by incubation of empty preformed liposomes with a gentamicin stock solution).

Figure 6 shows that externally bound gentamicin alone showed some anti-bacterial activity compared to the free gentamicin control. Liposomes containing gentamicin, but with external gentamicin removed showed bactericidal activity approximately equivalent to the liposomes with internal plus external gentamicin.

To test for fusogenicity and pH sensitivity of the liposomes, cells were incubated with bafilomicin A1 (100 nm; Sigma) during liposome treatment. This drug specifically blocks the endosomal membrane H^+-atpase pump, disabling the acidification process, and therefore any low pH-dependent liposome destabilization. The invasion assay was otherwise carried out as usual.

Figure 7 shows the results of this assay. DOPE:n-succinyl DOPE formulations were completely inhibited in their ability to kill intracellular bacteria (recombinant *S. typhimurium)* indicating that the mechanism of action of these carriers was dependant upon normal maturation of the endosome. In contrast, activity seen with DOPC:n-succinyl DOPE formulations in the absence of bafilomicin was reduced to a lesser extent. The DOPC formulation was not expected to undergo pH-mediated destabilization. The fact that DOPC:n-succinyl DOPE was efficacious in this model could indicate the presence of succinyl (and glutaryl) specific destabilization effects present within the endosomal environment.

C. Example 3: *In Vitro* Fusion Activity of Formulations

A description of the use of the fluorescent membrane assay to monitor fusion events has been already described (Struck, D., *et al., Biochemistry* **20**:4093-4099 (1981)). Briefly, liposomes that contained two fluorescent moieties that quench each other by resonance energy transfer when in close proximity were prepared. These vesicles were mixed with excess unlabeled liposomes of the same composition at different pHs. If fusion occurred, the liposome populations would fuse, the fluorescent probes would be effectively diluted in the membrane and dequenching would occur. Fusion was be monitored by an observed increase in fluorescence. For this assay, 100 nm donor vesicles of the formulations under examination were made containing 0.5 mol % of NBD-PE (*N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidyl ethanolamine, Avanti Polar Lipids) and Rh-PE (*N*-(lissamine rhodamine B sulfonyl)-dioleoyl phosphatidylethanolamine, Avanti Polar Lipids). Acceptor vesicles of the same composition as the donor but without the fluorescent molecules were also formulated. To a quartz cuvette (Hellma), the following was added: FBS (200 µL), donor liposomes (in 10 mM HBS) to 0.1 mM lipid, acceptor liposomes (in 10 mM HBS), to 1.0 mM, 150 mM saline (added first) to a final volume of 1.95 mL, and a magnetic (flea) stir bar. The cuvette was placed in an SLM Aminco-Bowman series II luminescence spectroscope with a stirring mechanism and heating mechanism (set to 37°C). After a two minute stabilization period, fluorescence emission at 535 nm (NBD fluorescence) was measured at the excitation wavelength of 445 nm, with a 4 nm slit width and an initial fluorescence set between 50 and 70% full scale. After a stable baseline was verified, 50 µL of acid solution (2.5 % (vol/vol) acetic acid plus 250 mM citrate-buffered saline pH 4.5) or dilutions thereof was added directly to the cuvette. Fluorescence was measured until a

plateau was achieved. Complete dequenching of the NBD was determined by the addition of 50 µL of 0.2 M $C_{12}E_8$ detergent. Data was processed using software provided by Aminco-Bowman. Percent NBD dequenching, equivalent to % fusion, was determined using the formula:

$$\% \text{ dequenching} = \frac{\text{acid induced fluorescence - initial fluorescence}}{C_{12}E_8 \text{ fluorescence - initial fluorescence}} \times 100\ \%$$

Figure 5 shows the results of this assay at four time points. Two formulations were found to fuse under acidic conditions in the presence of 10% serum, but the DOPE:n-succinyl DOPE formulation was found to have much greater fusion activity at earlier times than the control in the pH range from 4.5 to 5.0, the pH range of the late endosome/lysosome. No fusion was seen in the absence of serum for either formulation even down to pH as low as 3.0 (data not shown).

Although there are other conditions in the endosome that could lead to liposomal membrane destabilization, the above result indicates that the difference in fusion activity at endosomal pH could be an important factor.

D. Example 4: Flow Cytometry

To determine the efficiency of delivery of the liposome contents to cells, liposomes containing either the fluorescent compound calcein in self-quenching concentrations, or 5-sulfofluorescein diacetate (which becomes fluorescent when cleaved by cellular enzymes) were incubated with COS cells then analyzed by flow cytometry (FL1 detector; excitation 488 nm, emission 530±15 nm). For this assay, COS cells were used because of their ease in manipulation for flow cytometry compared with J774 cells. COS cells (ATCC Accession No. 1650) were grown overnight in DMEM-10% FBS at 37°C, 5% CO_2. Liposomes (DOPE:n-succinylDOPE:PEG (69.5:30:0.5) and DOPC:n-succinylDOPE:PEG (69.5:30:0.5)) containing either calcein in self-quenching concentrations (80 mM, Molecular Probes, Eugene, OR) or 5-sulfofluorescein diacetate (5-SFDA, 1 mg/mL, Molecular Probes) were added to COS cells ($1x10^5$/well in a 6-well plate) and incubated for 30 minutes. Cells were washed with PBS, trypsinized to detach, transferred to eppendorf tubes, washed twice with PBS and analyzed on a Becton Dickinson FACSort Flow Cytometer. At the same time, an identical experiment was performed with empty liposomes of the same formulation with small amounts (0.5 mol%) of NBD-PE to ascertain

the uptake of the lipid component. Analysis software used was Becton Dickinson FACScomp for cytometer calibration and CellQuest for sample analysis.

In both cases, uptake into the cell followed by degradation of the liposomes led to an increase in fluorescence. Figure 6 shows the results of these assays. The intracellular fluorescence of both calcein and 5-SFDA is higher in the case of the DOPE:-containing formulations. With calcein, the mean fluorescence per cell is 3454 arbitrary units (A.U.) compared to 501 units for DOPC formulations (approximately 7-fold higher), even though, as shown in panel B, the liposomal uptake for the DOPE formulation is only 3-fold greater for these cells. Panel C shows 5-SFDA fluorescence. The DOPE-containing formulation shows a fluorescence mean of 1054 A.U. compared to 437 A.U. for the DOPC formulation. These results were obtained although the lipid uptake is virtually identical (mean of 29 versus 26 A.U.). These data indicate that the DOPE-based formulation is more efficient than the DOPC-based formulation at delivering its contents into the cell.

The disclosures in this application of all articles and references, including patent documents, are incorporated herein by reference.

E. Example 5. Cholesterol-containing formulations

Liposomal formulations of gentamicin with the compositions DOPE:n-succinylDOPE:Cholesterol:PEG-C_{20} ceramide (35:30:30:5) and DOPE:n-succinylDOPE:Cholesterol:PEG-C_{20} ceramide (45:20:30:5) were manufactured by the process detailed in Example 1, and had similar antibacterial effects in the cell culture assays performed as in Example 2. These compositions were also extremely effective in an *in vivo* murine salmonellosis infection model.

WHAT IS CLAIMED IS:

1. A composition comprising of non-cationic liposome and a cationic drug wherein said liposome becomes fusogenic in an acidic environment.

2. The composition of claim 1, wherein said drug is within the interior of said liposome.

3. The composition of claim 1, wherein said cationic drug is an antibiotic.

4. The composition of claim 3, wherein said antibiotic is gentamicin.

5. The composition of claim 4, wherein said gentamicin is selected from the group consisting of gentamicin C_1, gentamicin C_2, gentamicin C_{1a}, and mixtures thereof.

6. The composition of claim 1, wherein said fusogenesis releases said drug from the liposome.

7. The composition of claim 1, wherein said liposome comprises an anionic lipid.

8. The composition of claim 7, wherein said anionic lipid has the general formula R-X, wherein R is selected from the group consisting of n-succinyl and n-glutaryl, n-dodecanoyl and mixtures thereof and wherein X is selected from the group consisting of phosphatidylethanolamines, ceramides and glycolipids.

9. The composition of claim 8, wherein X is phosphatidylethanolamine.

10. The composition of claim 9, wherein said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

11. The composition of claim 8, wherein said liposome further comprises a lipid selected from the group consisting of dioleoylphosphatidylethanolamine, diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine, dipalmitoylphosphatidylcholine,

dioleoylphosphatidylcholine, lysophosphatidylcholine, diacylphosphatidylcholine, and dioleoylphosphatidylserine, cholesterol and mixtures thereof.

12. The composition of claim 11, wherein said lipid is dioleoylphosphatidylethanolamine.

13. The composition of claim 12, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is from about 20% to 90%.

14. The composition of claim 13, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is about 70%.

15. The composition of claim 8, wherein said liposome comprises cholesterol.

16. The composition of claim 15, wherein the percentage of cholesterol is about 20% to about 50%.

17. The composition of claim 1, wherein said liposome comprises a PEG_n- lipid derivative, wherein n is selected from the group consisting of about 14 to 20 glycol units.

18. The composition of claim 17, wherein said liposome further comprises a lipid having the general formula R-X, wherein R is selected from the group consisting of n-succinyl and n-glutaryl, n-dodecanoyl and mixtures thereof and is selected from the group consisting of phosphatidylethanolamines, ceramides and glycolipids.

19. The composition of claim 18, wherein X is selected from the group consisting of phosphatidylethanolamines.

20. The composition of claim 19, wherein said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

21. The composition of claim 20, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is from about 35% to about 45%.

22. The composition of claim 15, wherein said liposome further comprises a lipid selected from the group consisting of dioleoylphosphatidylethanolamine,

diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, lysophosphatidylcholine, diacylphosphatidylcholine, and dioleoylphosphatidylserine, cholesterol and mixtures thereof.

23. The composition of claim 22, wherein said lipid is dioleoylphosphatidylethanolamine.

24. The composition of claim 22, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is from about 20% to about 90%.

25. The composition of claim 24, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is about 70%.

26. The composition of claim 22, wherein said liposome comprises cholesterol.

27. The composition of claim 26, wherein the percentage of cholesterol is from about 20% to about 50%.

28. The composition of claim 22, wherein said PEG_n- lipid derivative is PEG_n-ceramide.

29. The composition of claim 28, wherein said PEG_n-ceramide has an acyl chain of about 8 to about 20 carbon atoms.

30. The composition of claim 28, wherein said liposome has a molar percentage of PEG_n-ceramide from about 0.25 to 5.0.

31. The composition of claim 1, wherein said liposome comprises dioleoylphosphatidylethanolamine, R-dioleoylphosphatidylethanolamine, PEG-C_{20} ceramide and said drug is gentamicin, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl.

32. The composition of claim 31, wherein the ratio by weight of drug to liposome is 0.08 to 0.25.

33. The composition of claim 32, wherein the ratio by weight of drug to liposome is about 0.10 to 0.22.

34. A method of delivering a cationic drug into a cell to ameliorate a pathological condition, said method comprising encapsulating said drug in a non-cationic liposome, wherein said liposome becomes fusogenic in an acidic environment, and contacting said cell with said liposome, thereby delivering said drug into said cell.

35. The method of claim 34, wherein said drug is gentamicin.

36. The method of claim 35, wherein said gentamicin is selected from the group consisting of gentamicin C_1, gentamicin C_2, gentamicin C_{1a}, and combinations thereof.

37. The method of claim 34, wherein said pathological condition is a bacterial infection.

38. The method of claim 37, wherein pathological condition is caused by an intracellular pathogen.

39. The method of claim 37, wherein said bacterial infection is caused by the intracellular presence of a genus of bacteria selected from the group consisting of *Salmonella*, *Listeria*, and *Mycobacterium*.

40. The method of claim 34, wherein said liposome comprises an anionic lipid.

41. The method of claim 40, wherein said anionic lipid has the general formula R-X, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl and mixtures thereof and X is selected from the group consisting of phosphatidylethanolamines, ceramides and glycolipids.

42. The method of claim 41, wherein X is selected from the group consisting of phosphatidylethanolamines.

43. The method of claim 42, wherein said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

44. The method of claim 41, wherein said liposome further comprises a lipid selected from the group consisting of dioleoylphosphatidylethanolamine, diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, lysophosphatidylcholine, diacylphosphatidylcholine, and dioleoylphosphatidylserine, cholesterol and mixtures thereof.

45. The method of claim 44, wherein said liposome comprises dioleoylphosphatidylethanolamine.

46. The method of claim 45, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is from about 20% to 90%.

47. The method of claim 46, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is about 70%.

48. The method of claim 44, wherein said liposome comprises cholesterol.

49. The method of claim 48, wherein the percentage of cholesterol is from about 20% to about 50%.

50. The method of claim 34, wherein said liposome comprises a PEG_n-lipid derivative, wherein n is selected from the group consisting of about 14 to 20 glycol units.

51. The method of claim 50, further comprising an anionic lipid.

52. The method of claim 51, wherein said anionic lipid has the general formula R-X, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl and mixtures thereof and X is selected from the group consisting of phosphatidylethanolamines, ceramides and glycolipids.

53. The method of claim 52, wherein X is selected from the group consisting of phosphatidylethanolamines.

54. The method of claim 53, wherein said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

55. The method of claim 52, wherein said liposome further comprises a lipid selected from the group consisting of dioleoylphosphatidylethanolamine, diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, lysophosphatidylcholine, diacylphosphatidylcholine, and dioleoylphosphatidylserine, cholesterol and mixtures thereof.

56. The method of claim 55, wherein said lipid is dioleoylphosphatidylethanolamine.

57. The method of claim 56, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is from about 20% to 90%.

58. The method of claim 57, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is about 70%.

59. The method of claim 55, wherein said liposome comprises cholesterol.

60. The method of claim 59, wherein the percentage of cholesterol is from about 20% to about 50%.

61. The method of claim 50, wherein said lipid derivative is PEG_n-ceramide.

62. The method of claim 61, wherein said PEG_n-ceramide has an acyl chain of from about 8 to about 20 carbon atoms.

63. The method of claim 62, wherein said liposome has a molar percentage of PEG_n-ceramide from about 0.25 to 5.0.

64. The method of claim 34, wherein said liposome comprises dioleoylphosphatidylethanolamine, R dioleoylphosphatidylethanolamine, PEG-C_{20} ceramide and said drug is gentamicin, wherein R is selected from the group consisting of n-succinyl, n-glutaryl, and n-dodecanoyl.

65. The method of claim 64, wherein the ratio by weight of drug to liposome is 0.8 to 0.25.

66. The method of claim 65, wherein the ratio by weight of drug to liposome is about 0.10 to 0.22.

67. A pharmaceutical composition comprising a non-cationic liposome-encapsulated cationic drug, wherein said liposome becomes fusogenic in an acidic environment, and a pharmaceutically acceptable carrier.

68. The pharmaceutical composition of claim 67, wherein said cationic drug is an antibiotic.

69. The pharmaceutical composition of claim 68, wherein said antibiotic is gentamicin.

70. The pharmaceutical composition of claim 69, wherein said gentamicin is selected from the group consisting of gentamicin C_1, gentamicin C_2, gentamicin C_{1a}, and mixtures thereof.

71. The pharmaceutical composition of claim 67, wherein said liposome comprises an anionic lipid.

72. The pharmaceutical composition of claim 71, wherein said anionic lipid has the general formula R-X, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl and mixtures thereof and X is selected from the group consisting of phosphatidylethanolamines, ceramides and glycolipids.

73. The pharmaceutical composition of claim 72, wherein X is selected from the group consisting of phosphatidylethanolamines.

74. The pharmaceutical composition of claim 73, wherein said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

75. The pharmaceutical composition of claim 72, wherein said liposome further comprises a lipid selected from the group consisting of dioleoylphosphatidylethanolamine, diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine,

dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, lysophosphatidylcholine, diacylphosphatidylcholine, and dioleoylphosphatidylserine, cholesterol and mixtures thereof.

76. The pharmaceutical composition of claim 75, wherein said lipid is dioleoylphosphatidylethanolamine.

77. The pharmaceutical composition of claim 76, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is from about 20% to 90%.

78. The pharmaceutical composition of claim 77, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is about 70%.

79. The pharmaceutical composition of claim 75, wherein said liposome comprises cholesterol.

80. The pharmaceutical composition of claim 79, wherein the percentage of cholesterol is from about 20% to about 50%.

81. The pharmaceutical composition of claim 67, wherein said liposome further comprises a PEG_n-lipid derivative, wherein n is selected from the group consisting of about 14 to 20 glycol units.

82. The pharmaceutical composition of claim 81, wherein said liposome further comprises an anionic lipid.

83. The pharmaceutical composition of claim 82, wherein said anionic lipid has the general formula R-X, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl and mixtures thereof and X is selected from the group consisting of phosphatidylethanolamines, ceramides and glycolipids.

84. The pharmaceutical composition of claim 83, wherein X is selected from the group consisting of phosphatidylethanolamines.

85. The pharmaceutical composition of claim 83, wherein said phosphatidylethanolamine is dioleoylphosphatidylethanolamine.

86. The pharmaceutical composition of claim 83, wherein said liposome further comprises a lipid selected from the group consisting of

dioleoylphosphatidylethanolamine, diacylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine, distearoylphosphatidylethanolamine, lysophosphatidylethanolamine, palmitoyloeioylphosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, lysophosphatidylcholine, diacylphosphatidylcholine, and dioleoylphosphatidylserine, cholesterol and mixtures thereof.

87. The pharmaceutical composition of claim 86, wherein said lipid is dioleoylphosphatidylethanolamine

88. The pharmaceutical composition of claim 87, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is from about 20% to 90%.

89. The pharmaceutical composition of claim 88, wherein the percentage (mol/mol) of dioleoylphosphatidylethanolamine is about 70%.

90. The pharmaceutical composition of claim 86, wherein said liposome comprises cholesterol.

91. The pharmaceutical composition of claim 90, wherein the percentage of cholesterol is from about 20% to about 50%.

92. The pharmaceutical composition of claim 81, wherein said PEG_n-lipid derivative is PEG_n-ceramide.

93. The pharmaceutical composition of claim 91, wherein said PEG_n-ceramide has an acyl chain from about 8 to about 20 carbon atoms.

94. The pharmaceutical composition of claim 93, wherein said liposome has a molar percentage of PEG_n-ceramide from about 0.25 to 5.0.

95. The pharmaceutical composition of claim 67, wherein said liposome comprises dioleoylphosphatidylethanolamine, R-dioleoylphosphatidylethanolamine, PEG C_{20} ceramide and said drug is gentamicin, wherein R is selected from the group consisting of n-succinyl, n-glutaryl and n-dodecanoyl.

96. The pharmaceutical composition of claim 95, wherein the ratio by weight of drug to liposome is 0.08 to 0.25.

97. The pharmaceutical composition of claim 96, wherein the ratio by weight of drug to liposome is about 0.10 to 0.22.

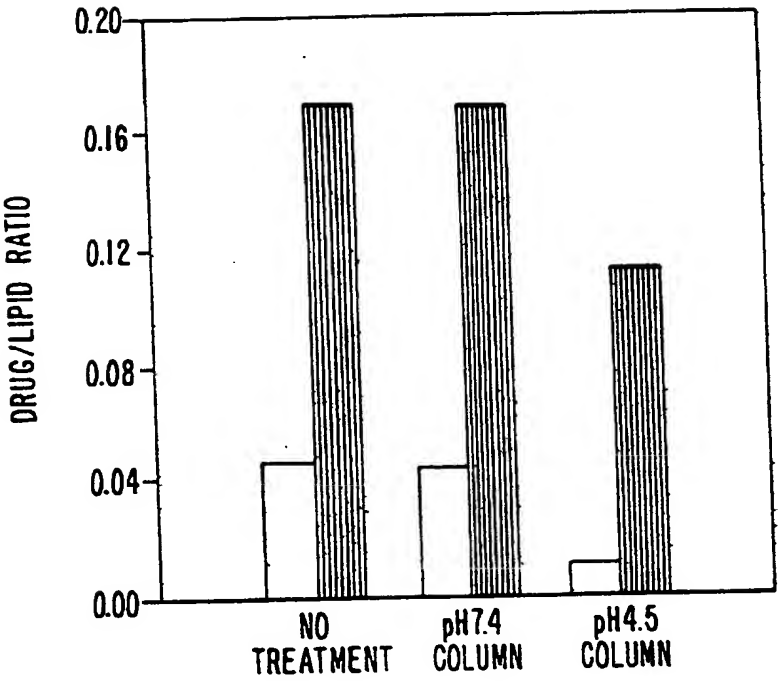
0.20
0.16
0.12
0.08
0.04
0.00
DRUG/LIPID RATIO
NO TREATMENT
pH7.4 COLUMN
pH4.5 COLUMN

FIG. 1.

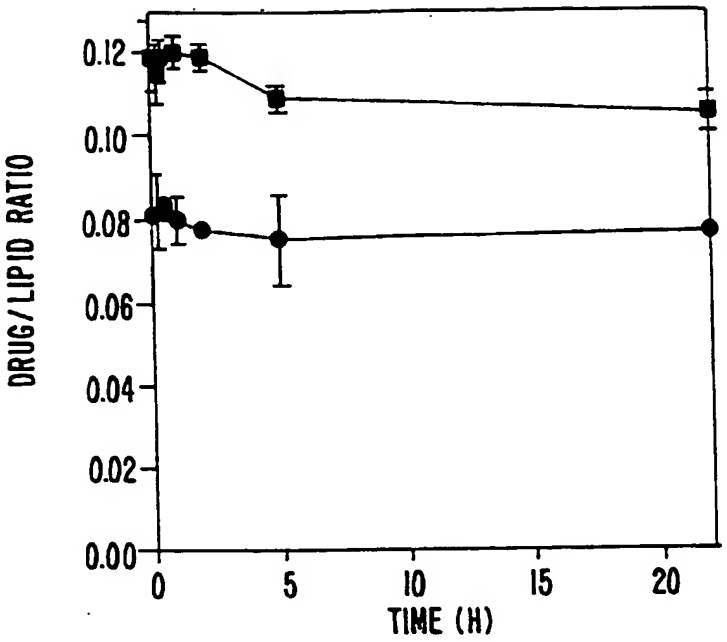
DRUG/LIPID RATIO
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
TIME (H)

FIG. 2A.

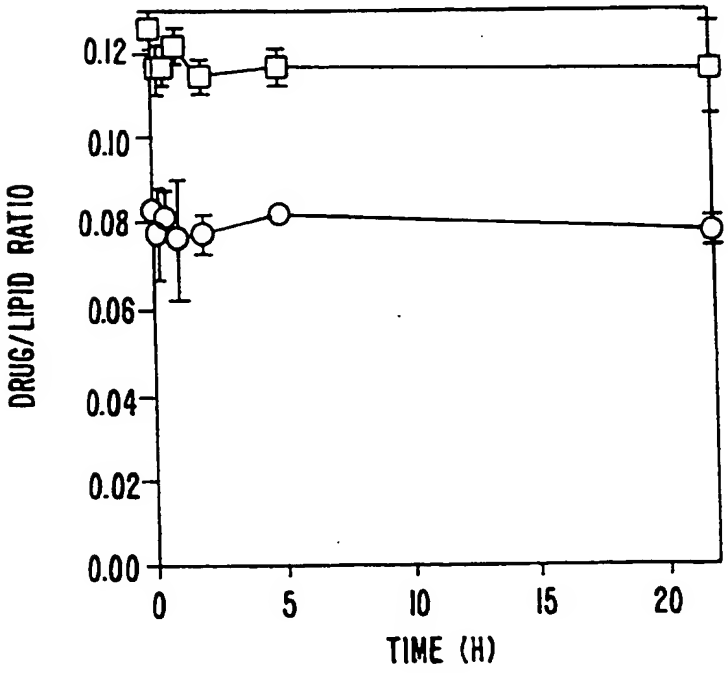
DRUG/LIPID RATIO
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
TIME (H)

FIG. 2B.

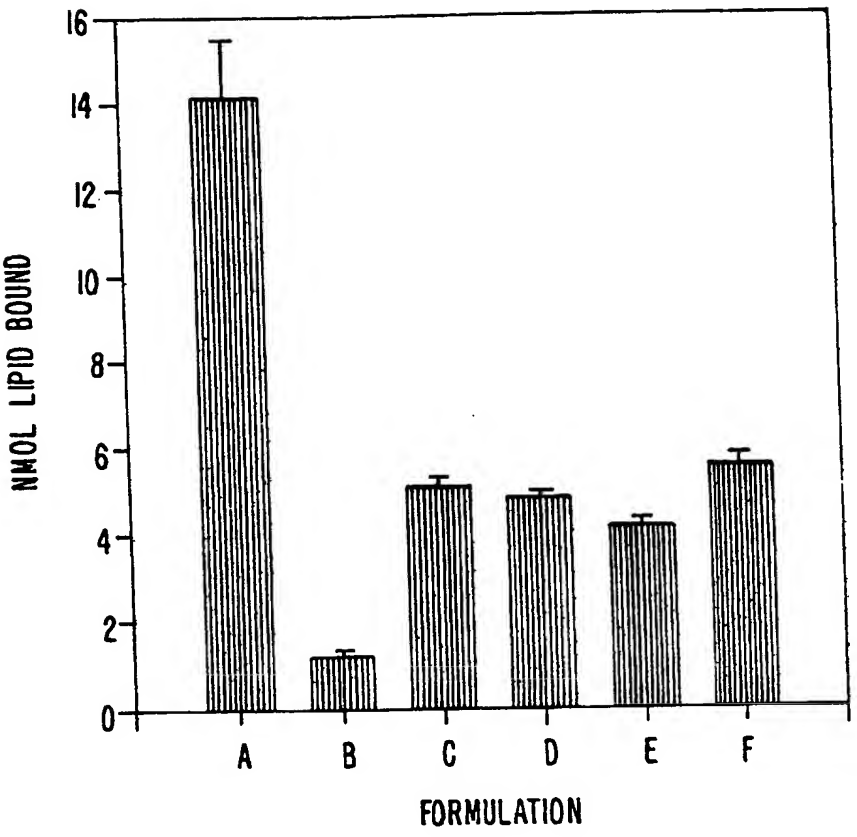

FIG. 3.

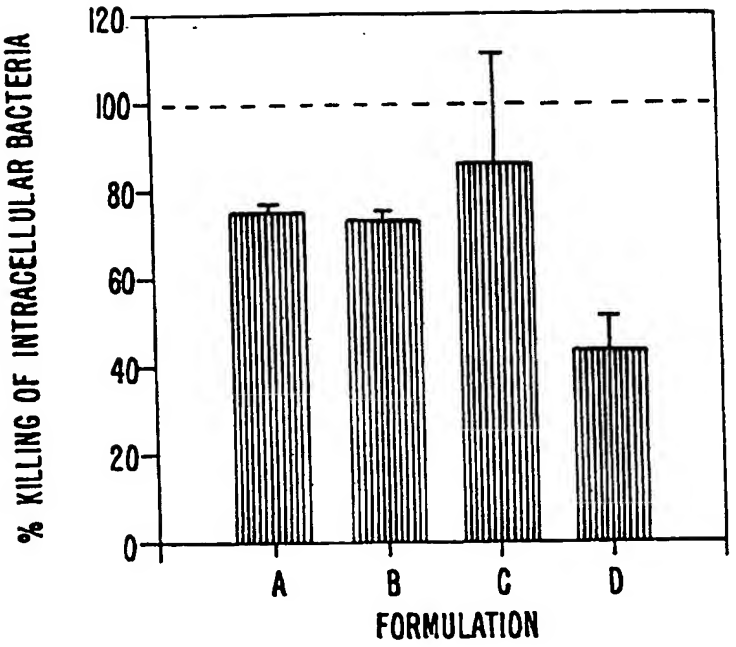
% KILLING OF INTRACELLULAR BACTERIA
120
100
80
60
40
20
0
A
B
C
D
FORMULATION

FIG. 4A.

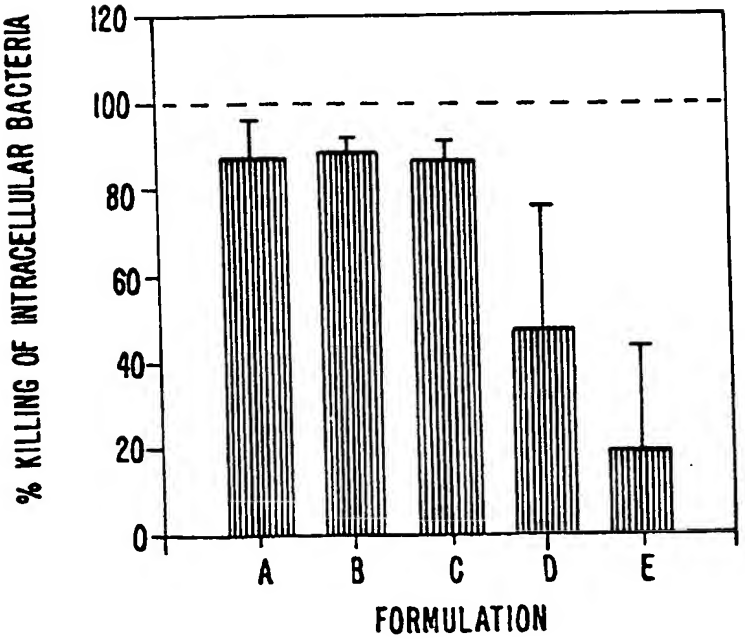
% KILLING OF INTRACELLULAR BACTERIA
120
100
80
60
40
20
0
A
B
C
D
E
FORMULATION

FIG. 4B.

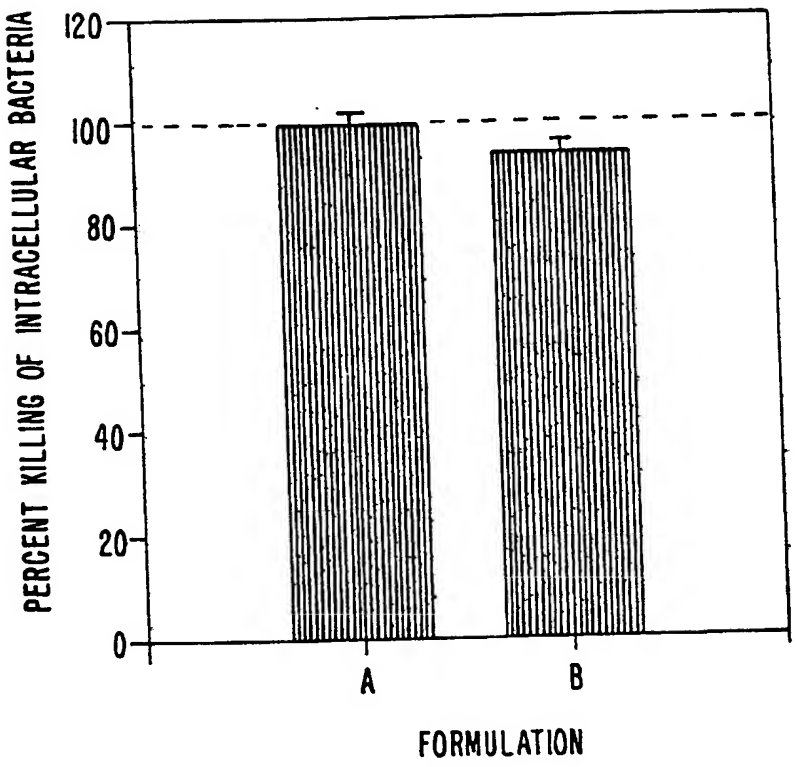
PERCENT KILLING OF INTRACELLULAR BACTERIA
120
100
80
60
40
20
0
A
B
FORMULATION

FIG. 5.

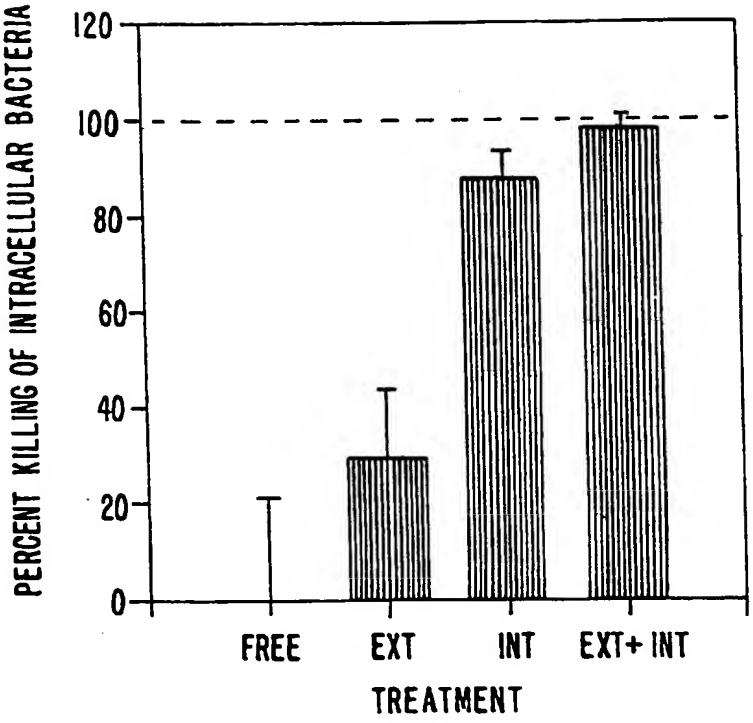
PERCENT KILLING OF INTRACELLULAR BACTERIA
120
100
80
60
40
20
0
FREE
EXT
INT
EXT+ INT
TREATMENT

FIG. 6.

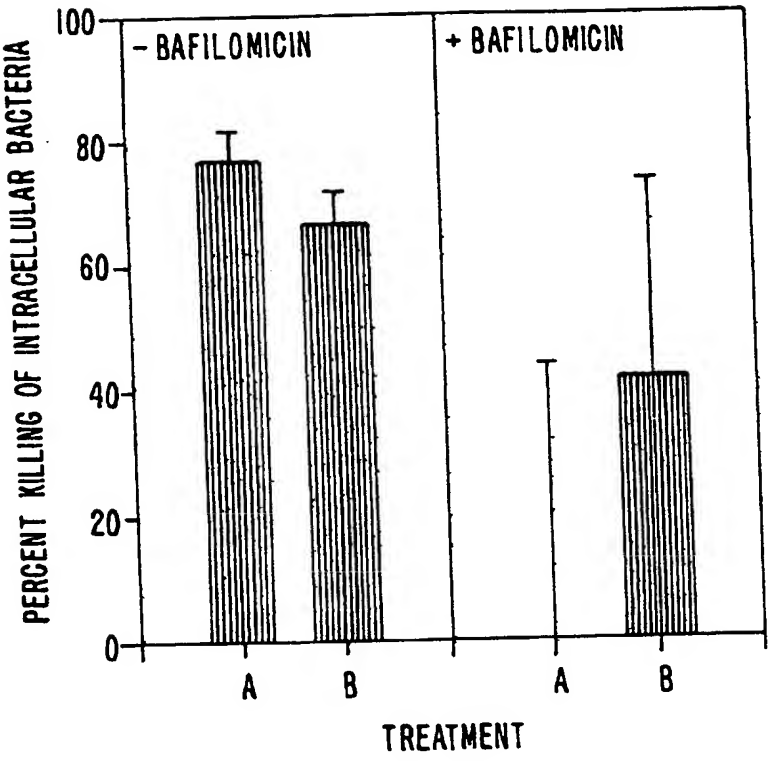
100
80
60
40
20
0
- BAFILOMICIN
+ BAFILOMICIN
PERCENT KILLING OF INTRACELLULAR BACTERIA
A
B
A
B
TREATMENT

FIG. 7.

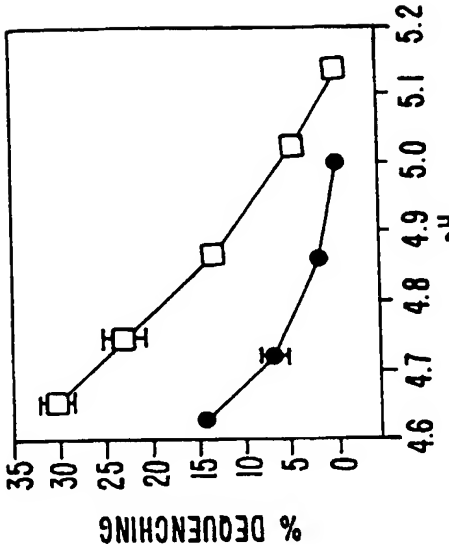

FIG. 8B.

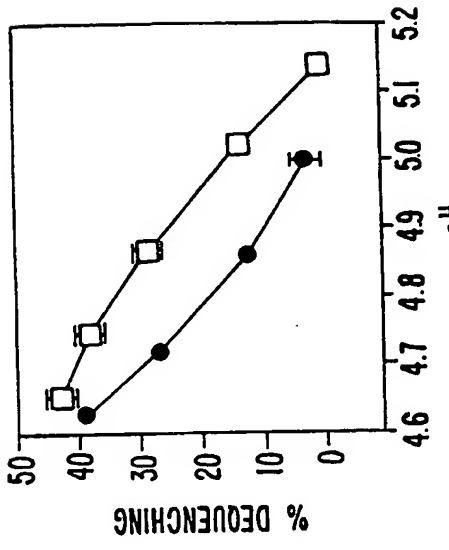

FIG. 8D.

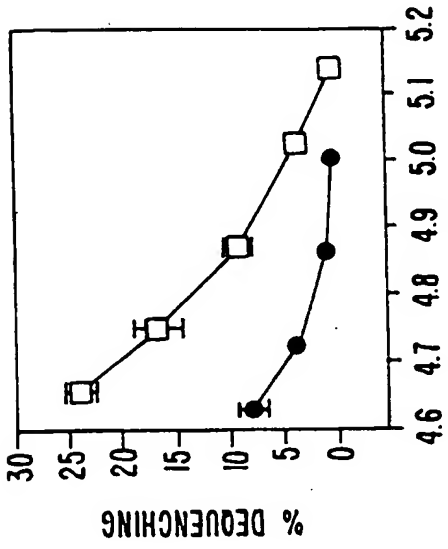

FIG. 8A.

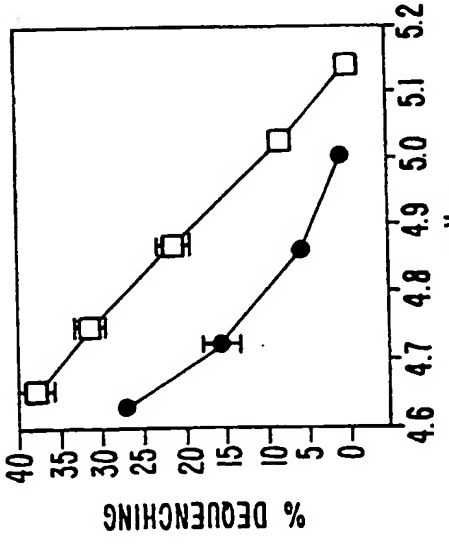

FIG. 8C.

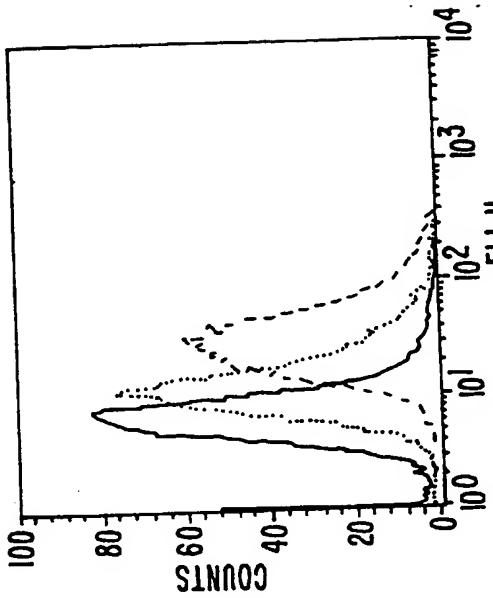

FIG. 9B.

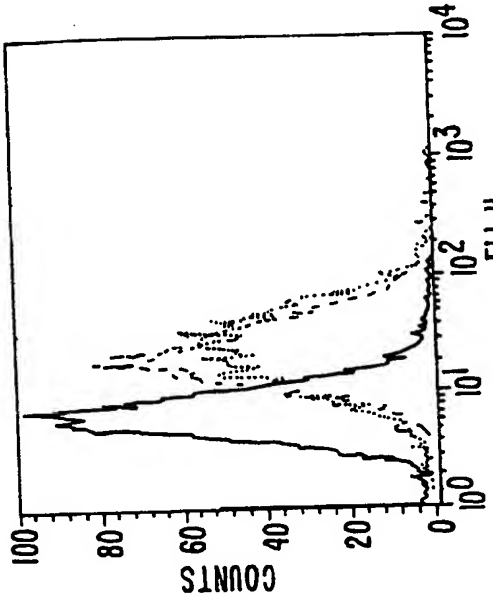

FIG. 9D.

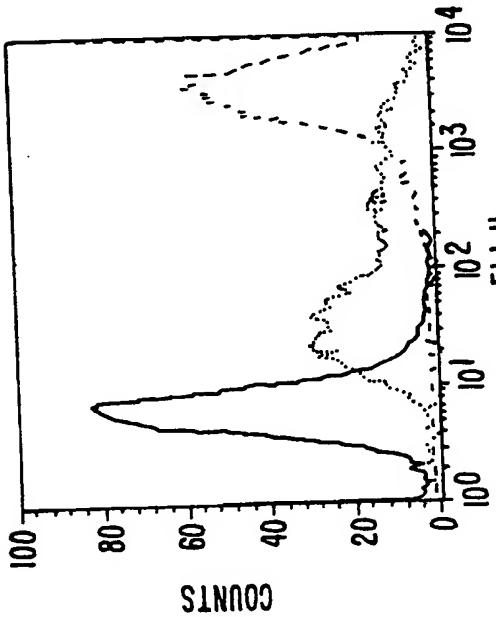

FIG. 9A.

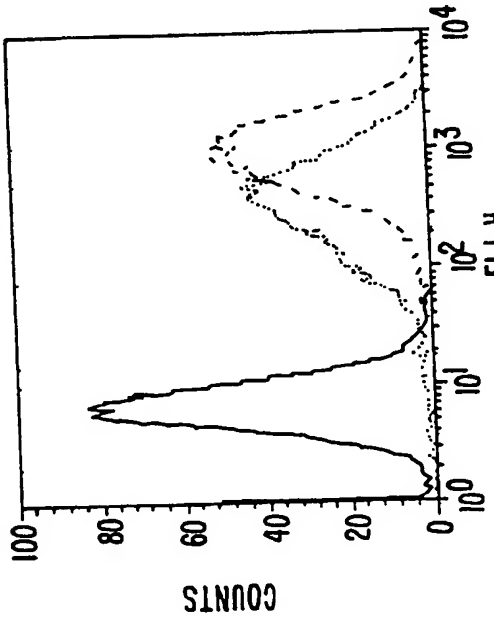

FIG. 9C.

INTERNATIONAL SEARCH REPORT

national Application No
PCT/CA 98/01154

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MORGAN, J. R.: "Preparation and properties of liposome-associated gentamicin" CURR. CHEMOTHER. INFECT. DIS., PROC. INT. CONGR. CHEMOTHER., 11TH, vol. 1, 1980, pages 680-1, XP002100260 see the whole document	1-4,34, 35
A	WO 96 10392 A (THE UNIVERSITY OF BRITISH COLUMBIA) 11 April 1996 see the whole document	1-97
A	WO 97 38010 A (THE UNIVERSITY OF BRITISH COLUMBIA) 16 October 1997 see the whole document	1-97

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 1999	28/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ventura Amat, A

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

national Application No
PCT/CA 98/01154

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9610392 A	11-04-1996	AU 3559995 A	26-04-1996
		CA 2201121 A	11-04-1996
		EP 0792142 A	03-09-1997
		JP 10506395 T	23-06-1998
WO 9738010 A	16-10-1997	AU 2284697 A	29-10-1997
		CA 2252055 A	16-10-1997